Identifying potential new stem cell biomarkers for prostate cancer

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ABSTRACT

Few biomarkers have been identified for prostate cancer diagnosis/prognosis and there are clinical difficulties in distinguishing between relapsing and non-relapsing tumours. Therefore, identifying new biomarkers for prostate cancer has become a priority. Recently, potential stem cells found within a tumour have received a lot of attention and it is thought that these cells may have a role in cancer initiation, progression and drug resistance. The major goal of this study was to focus on proteins linked to stem cells, cellular differentiation and/or tumour formation and progression and establish if they are differentially expressed between normal and malignant prostate tissues and/or between samples with different clinical features, including relapsing vs non-relapsing tumours.

Eight potential biomarkers were identified using literature searching. These were β-catenin, NDRG1, ABCG2, ALDH1A1, RS1, Sox7, Sall4 and Zscan4. Their expression was evaluated by immunohistochemistry using 39 prostate tissue samples from a Bath cohort and the staining was then repeated using a much larger tissue microarray cohort that consisted of 96 cases. The data from both cohorts was then tested for association with different clinical features. Finally, the expression of three of the potential biomarkers, Sox7, Sall4 and Zscan4 was further validated by staining tissues from both cohorts with either a second independent antibody and /or for the biomarkers' mRNA using RNAscope®.

β-catenin, Sox7, ABCG2, membranous and cytoplasmic NDRG1 staining was found to be reduced in prostate cancer samples, whereas, Zscan4, nuclear NDRG1 and Sall4 staining was increased. There was a negative association between β-catenin, Sox7, ABCG2 and Sall4 staining and increasing Gleason grade, whereas, nuclear NDRG1 staining was positively associated with Gleason grade. There was also a negative association between Sox7, ABCG2 and stromal ALDH1A1 staining and biochemical relapse. In contrast, there was no significant association between RS1 staining and prostate cancer clinical features. Therefore, this data identified seven proteins that may play a role in prostate tumour formation and/or aggressiveness and three that may be implicated in PCa relapse. These candidates warrant further investigation to understand their functions and establish if they could represent potential diagnostic/prognostic biomarkers for prostate cancer.

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LIST OF ABBREVIATIONS AND ACRONYMS

AACR	American Association for Cancer Research
ABCG2	ATP binding cassette group 2
ACD	Advanced cell diagnostic
AJCC/ UICC	American joint committee on cancer / International Union against cancer
ALDH	aldehyde dehydrogenase
ALDH1A1	Aldehyde hydrogenase 1 family member A1
AMACR	Alpha methyl acyl coenzyme A racemase
AP	Alkaline phosphatase
APTS	(3 Aminopropyle) triethoxysilane
AR	Androgen receptor
AREs	Androgen response elements
Axin-2	Axin inhibition protein -2
Bax	Bcl2 associated X
Bcl-2	B-cell lymphoma 2
BCRP	Breast cancer resistance protein
Bmi-1	BMI1 polycomb ring finger oncogene
BPH	Benign prostate hyperplasia
BSA	Bovine Serum Albumin
C-Myc	Myelocytomatosis viral oncogene homolog
CRPC	Castration-resistant prostate cancer
CSCs	Cancer stem cells
CZ	Central zone
DAB	Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic Acid
EBV	Epstein-Barr virus
EDTA	Ethylene diamine tetra acetic Acid
ELISA	Enzyme-linked Immunosorbent Assay
EMT	Epithelial-Mesenchymal Transition

ERG	V-Ets Avian Erythroblastosis Virus E26 Oncogene Homolog
ERK	Extracellular signal-regulated kinase
ESC	Embryonic Stem Cell
ETS	E26 transformation-specific
FADD	Fas-Associated protein with Death Domain
FFPE	Formalin-fixed paraffin-embedded
FGF	Fibroblast growth factor
FISH	Fluorescent In Situ Hybridisation
Foxa 2	Forkhead transcription factor
FOXP3	Forkhead Box P3
FR	Fast red
FRAT1	Frequently rearranged in advanced T- cell
GSK3B	Glycogen synthase kinase 3 beta
GSTP1	Glutathione S -transferase π
H&E	Haematoxylin and Eosin
H2O2	Hydrogen peroxide
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
HGPIN	High grade prostate intraepithelial neoplasia
HMWCK	High molecular weight cytokeratin
HRP	Horseradish peroxidase
HSP	Heat shock protein
ICM	Inner cell mass
IHC	Immunohistochemistry
Ips	pluripotent stem cells
ISH	In situ hybridization
LEF	Lymphoid enhancer-binding factor
LRP5/6	Low –density lipoprotein receptor-related protein s/6
M	metastasis
Max	Myc associated factor X
mRNA	Messenger ribonucleic acid
MSP	Methylation- specific PCR
N	Lymph node

NDRG1	N-myc downstream regulated gene 1
NGS	Normal Goat Serum
NH3	Ammonia
NHS	National health service
NKX3.1	Homeobox NKX3.1 gene
NP	Normal prostate
NSCLC	non – small cell lung cancer cell lines
OCT4	Octamer-binding protein 4
P53	Phosphoprotein 53
PAK4	P21- Activated Kinase 4
PAP	prostate acid phosphatase
PBS	Phosphate-buffered saline
PBST	Phosphate buffer saline with 0.05 tween 20
PCa	Prostate cancer
PCSCs	Prostate cancer stem cells
PI3K	Phosphoinositide 3-kinase
PIA	Proliferative inflammatory atrophy
PIN	prostatic intraepithelial neoplasia
PIP2	Phosphatidylinositol 4,5 phosphate
PIP3	phosphatidylinositol 3, 4, 5 triphosphates
PRC	Polycomb repressor complex
PSA	prostate-specific antigen
PSCs	Prostate stem cells
PTEN	Phosphatase and tensin homolog
PZ	Peripheral zone
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RA	Retinoic acid
RAP 1	Repressor –activator protein 1
REC	Research ethics committee
RNA	Ribonucleic acid
RNA-Seq	Ribonucleic acid sequencing
RS1	Retinoschisis 1
RT	Regulated transcription

RUH	Royal United Hospital
Sall4	Sall-like 4
Sca-1	Stem cell antigen 1
SCs	Stem cells
siRNA	Small interfering ribonucleic acid
Sox2	Sex-determining region Y box 2
Sox7	Sex-determining region Y box 7
SPOP	Speckle-type POZ protein
SRC	Steroid receptor coactivator
SV	Seminal vesicle
T	Primary tumour size
TA	Transiently amplifying
TBP	TATA box binding protein
TCF	T cell factor
TCF4	Transcription Factor 4
TFIIF	Transcription factor IIF
TLE2	Transduction –like enhancer of split 2
TMA	Tissue microarray
TMPRSS-ERG	Transmembrane protease, serine 2- ETS- regulated gene
TNM	Tumor-Nodes- Metastasis
TRF1	Telomere repeat binding factor
TRIS	Tri-sodium citrate
TURP	transurethral resection of the prostate
TZ	Transition Zone
Wnt	Wingless-integration site
Zscan4	Zinc finger and SCAN domain containing 4
α2β1	α2β1 integrin

CHAPTER ONE
INTRODUCTION

1. Introduction

Prostate cancer (PCa) is a heterogeneous disease and represents a global healthcare issue that is mostly a diagnosis of elderly men (Dunn and Kazer, 2011). A few diagnostic and prognostic biomarkers have been identified for PCa, including prostate-specific antigen (PSA) (Qu *et al.*, 2014). However, there is a need for more specific and/or sensitive biomarkers in PCa diagnosis and especially for measuring PCa prognosis, for example, to distinguish between relapsing and non-relapsing cases. Cancer stem cells (CSCs) have been linked to PCa relapse and so analysis of expression of proteins that have been linked to stem cells (SCs) represents an interesting new avenue for future biomarker studies. This thesis will take this approach and examine the expression of proteins linked to SCs in PCa to try and identify potential new biomarkers for the disease.

This introduction will describe the structure and cell types of the normal and malignant prostate, the diagnostic methods for PCa, and the main molecular processes that are involved in PCa formation and progression and finally provide information about SCs and CSCs in prostate tissue.

1.1 Normal prostate

This section will provide information about the anatomy, physiology and histology of the normal prostate (NP).

1.1.1 Prostate anatomy

The prostate is the largest accessory gland in the male reproductive system. It is a glandular organ surrounding the neck of the urinary bladder and urethra and the normal weight of the adult prostate is about twenty grams (Epstein, 2010). The prostate consists of glandular and non-glandular regions. The glandular region can be divided into three zones: peripheral, central and transitional zones (Figure1.1) and most pathological lesions of prostate arise from the glandular region. For example, 75% of PCa develop in the peripheral zone and less frequently in the transitional and the central zones (McNeal, 1984; Applewhite *et al.*, 2001; Hammerich *et al.*, 2008; Mocarska *et al.*, 2014). The non-glandular region consists of a single zone, called the anterior fibromuscular stroma (McNeal, 1984) (Figure1.1). The

prostate is also surrounded by a fibromuscular tissue layer, called the prostate capsule (Fine and Reuter, 2012).

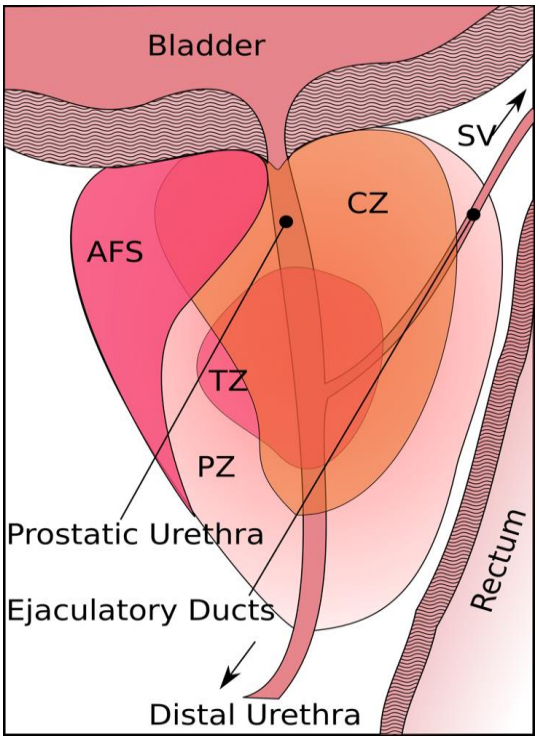

Figure 1.1 Prostate anatomy as described by McNeal. The NP consists of several distinct regions, including three glandular regions: the peripheral zone (PZ), transition zone (TZ) and central zone (CZ). In addition to a non-glandular region of anterior fibromuscular stroma (AFS). Through the ejaculatory duct (labelled), Seminal vesicles (SV) secrete their fluid into the urethra. Adapted from (McNeal, 1984; Sharpe, 2016).

1.1.2 Prostate physiology

The major role of the prostate gland is the production of an alkaline fluid that forms part of the seminal fluid and promotes sperm movement and activity (Frick and Aulitzky, 1991; Abate-Shen and Shen, 2000; Khan, 2011; Bhavsar and Verma, 2014). In addition, prostate muscles assist in discharging the semen at the time of ejaculation (Khan, 2011). Previous studies have reported that the prostate gland represents a manufacturer of PSA which plays a role in dissolving the seminal clots and promoting sperm motility in the female reproductive tract as well as to nourish sperm (Dunn and Kazer, 2011; Khan, 2011). The prostate can be regulated by dihydrotestosterone which is normally produced from a key male testicular hormone, testosterone in the peripheral zone of the prostate (Carson and Rittmaster, 2003).

1.1.3 Prostate histology

The histological architecture of the prostate glands shows that there are multiple epithelial glands surrounded by abundant fibromuscular stroma. The normal epithelial-stromal- ratio in human prostate tissue is 1:2 (Prajapati *et al.*, 2013).

The epithelial layer of the prostate contains three cell types; basal, secretory (luminal), and neuroendocrine cells (Signoretti and Loda, 2007; Goldstein *et al.*, 2010a) (Figure 1.2). Basal cells are small flattened or cuboidal undifferentiated cells located on the basement membrane of the prostate gland, these cells don't have a secretion action (Lang *et al.*, 2009b). The second type of epithelial cells that form the majority of cells in the epithelium of the prostate gland, is called luminal cells and these cells are fully differentiated columnar cells and have the ability to secrete materials into a glandular lumen of the prostate, including PSA and prostate acid phosphatase (PAP) (McNeal, 1984; Lang *et al.*, 2009b). In addition, there is a small population of fully differentiated cells located in the basal layer called neuroendocrine "endocrine-paracrine" which play a role in secreting serotonin (McNeal, 1984; Bonkhoff *et al.*, 1995; di Sant'Agnese and Cockett 1996) and a number of neuropeptides such as chromogranin, calcitonin family peptides, parathyroid-like hormone and somatostatin (Di Sant'Agnese and Cockett, 1996; Parimi *et al.*, 2014). Surrounding the epithelial cells are several types of cells in the stromal layer of the prostate, including smooth muscle cells, fibroblasts, and myofibroblasts (Takao and Tsujimura, 2008) (Figure 1.2).

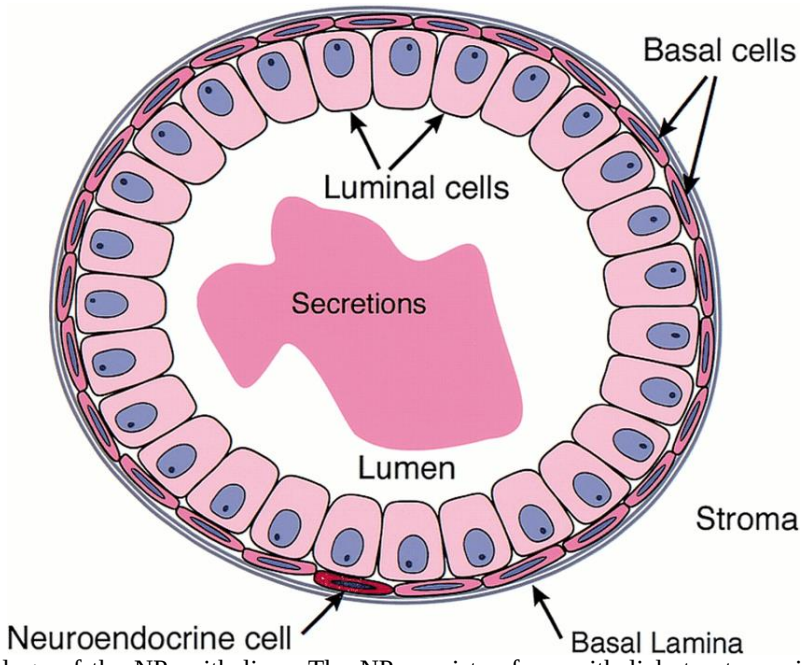

Figure 1. 2 Histology of the NP epithelium. The NP consists of an epithelial structure with three types of epithelial cells (Basal, Luminal and Neuroendocrine cells), a basement membrane and surrounding stroma. In

the ductal lumen, there are secretary substances (labelled) such as zinc, citrate, and PSA that are produced by luminal cells. Histologically, both basal and neuroendocrine cells are normally found in the basal cell layer and cannot be distinguished microscopically. Adapted from (Abate-Shen and Shen, 2000).

There are three most common pathological disorders which affect the prostate glands, including prostatitis (inflammation of the prostate), benign prostate hyperplasia (BPH) (enlargement of the prostate gland) and cancers (Epstein, 2010). The current study will focus on carcinoma of the prostate.

1.2 Prostate cancer

1.2.1 Introduction

PCa can broadly be defined as an abnormal growth that usually begins in the prostate glands. In the United Kingdom, PCa represents the second most frequent malignancy and is the second highest cause of death in males after lung cancer (Lang *et al.*, 2009b; Kirby, 2012). Adenocarcinoma is the most common type of PCa which represents more than 90% of PCa, and it originates from the glandular regions of the prostate gland (Bagnall, 2014; Dunn and Kazer, 2011). Other types of carcinoma are also observed but are rare in the prostate gland, including small cell (neuroendocrine) carcinoma, squamous cell carcinoma, urothelial cell carcinoma and sarcomatoid carcinoma (Li and Wang, 2016).

1.2.2 Risk factors of prostate cancer

Although the main causes of PCa are still being fully established, several risk factors have been linked to the development and progression of PCa (Bostwick *et al.*, 2004), including age (Kirby, 2012), race (Dunn and Kazer, 2011), family history (Kirby, 2012), hormones and diets (Bostwick *et al.*, 2004).

Previous studies found over half (54%) of PCa cases are diagnosed in males over the age of 75, whereas, this risk generally is reduced in males under the age of 50 (Bostwick *et al.*, 2004) suggesting PCa is an illness of elderly men. This may be because of the slow-growing nature of PCa, as well as the activity of the immune system which reduces with advanced age. In addition, Ben-Shlomo *et al.* identified an increased risk of PCa in black UK males compared to those with white skin, however, this risk is still lower than black American males (Ben-Shlomo *et al.*, 2008). Another study by Metcalfe *et al.* found that Asian men have a higher risk of PCa after they emigrate to the United Kingdom compared to the general population (Metcalfe *et al.*, 2008). This evidence suggests that race plays a role in PCa

development. Family history is also found to play a role in increasing the risk of PCa development in the next generation. A previous report found that approximately 9% of PCa cases have an inherited basis and the risk of PCa rises 2.5 fold for men who have a family history with PCa (Kirby, 2012). This evidence suggests that this difference may be due to genetics and environmental factors.

Hormonal changes may promote the growth of PCa and it has been found that androgens have a significant role in the alteration of PCa development. PCa progression from preclinical to clinical form may occur as a result of androgen metabolism changes (Bostwick *et al.*, 2004). Another study found that testosterone has an essential role in the progression of PCa and castration of men plays a role in preventing PCa progression (Kirby, 2012). Diet is also found to be implicated in PCa development. For example, fat intake, particularly polyunsaturated fat, has a positive correlation with the rate of PCa development and its mortality rate. The reasons for this are proposed to be that fat induces changes in the hormone profile, along with metabolic effects such as a generation of protein or DNA reactive intermediate due to increased oxidative stress (Bostwick *et al.*, 2004). In addition, high consumption of red meat increases the risk of PCa, this might be due to increasing the carcinogenic substance during cooking the meat at a high temperature (Khan, 2011). The risk of PCa also increases in people who consume egg and foods which have sources of egg (Richman *et al.*, 2011). The reason for this correlation may be that eggs contain a high cholesterol concentration, which represents a precursor for androgen production and may promote the development of tumours due to its effect on steroidogenesis (Pelton *et al.*, 2012).

1.2.3 Steps in the development of prostate cancer

PCa grows slowly and is normally a disease of elderly males over the age of 50 (Leitzmann and Rohrmann, 2012). However, PCa is also identified in young men (Salinas *et al.*, 2014), suggesting PCa progression and the initiation of pre-neoplastic disorders may develop in the early stage of life.

Proliferative inflammatory atrophy (PIA) and prostatic intraepithelial neoplasia (PIN) have proposed to be potential precursor lesions to PCa (Woenckhaus and Fenic, 2008; Bostwick and Cheng, 2012). PIA is an atrophic lesion in the prostate architecture marked by increased proliferation, reduced apoptotic rate, detection of molecular-biological abnormalities specific for oxidative stress or malignancy (Woenckhaus and Fenic, 2008), and increased

inflammatory cells in both epithelial and stromal prostate regions (De Marzo *et al.*, 1999; DeMarzo *et al.*, 2003). PIA normally occurs in the peripheral prostate zone that represents the area in which most clinically important prostatic adenocarcinomas develops (De Marzo *et al.*, 1999). The inflammatory nature of PIA could potentially promote carcinoma either directly or indirectly through first developing into high grade PIN (HGPIN), which subsequently progresses to carcinoma (De Marzo *et al.*, 1999; DeMarzo *et al.*, 2003). However, the direct evolving from PIA to carcinoma is thought to be rare (Epstein, 2009).

PIN is thought to be another precursor of PCa development and to represent an intermediate stage between BPH and PCa (Bostwick and Cheng, 2012). PIN arises primarily from the peripheral zone of the prostate and the cytological features of the lesions are similar to those of PCa (Sakr and Grihnon, 1999). The histological feature of PIN is characterised by the presence of luminal epithelial hyperplasia, enlarged nuclei and nucleoli, the partial absence of basal cell layers, basement membrane (Jaworska *et al.*, 2015) and stromal reactivity (Packer and Maitland, 2016). In addition to the similarities to PCa, the histological features of PIN resemble benign glands with a basophilic appearance because of enlarged, crowded nuclei and increased nuclear to cytoplasmic ratio (Epstein, 2009). Based on the histological architecture and cytologic features, two different grades of PIN, low and high grade have been identified (Ayala and Ro, 2007; Bostwick and Cheng, 2012; Tolkach and Kristiansen, 2018). HGPIN is a potential source of PCa (Ayala and Ro, 2007; Bostwick and Cheng, 2012; Tolkach and Kristiansen, 2018). However, it has been found that not all PCa arises from HGPIN (Epstein, 2009).

There are a number of significant changes which are identified during the transformation of prostate cells from NP to PIN to carcinoma, including histological and molecular changes (Packer and Maitland, 2016). Firstly, the basal cell layer and basement membrane normally present within the NP gland. This histological architecture, however, is disrupted by the disappearance of the basal cell layer and basement membrane of the prostate gland either partially in a PIN or completely in PCa (Epstein, 2009; Liu *et al.*, 2009). Secondly, PCa tissues are also histologically characterized by hyperproliferation of the secretory cells (Goldstein *et al.*, 2010a) and increased immune cell infiltration (Packer and Maitland, 2016).

PCa patients are normally initially treated with surgery, a radical prostatectomy, or radiotherapy. This is successful for many patients, but roughly a third will suffer relapse (Tan *et al*., 2015b; Xie *et al*., 2014), which is defined as an increase in the PSA level in the

blood following the surgery or radiation treatment. Relapsing patients are often treated with hormonal therapy which is normally based on blockage of androgens by medicines that prevent androgen production and/or block the function of androgen receptor AR (Tan *et al*., 2015b), but the tumours can become resistant to the treatment and progress to a clinical condition that is called castration-resistant PCa (CRPC) (Tan *et al*., 2015b). This has previously been named as a hormonal refractory or androgen-independent PCa. CRPC patients will be shifted to second-line treatments, including chemotherapy such as docetaxel (Tan *et al.,* 2015b). However, chemotherapy is a non-curable treatment for the majority of CRPC patients (Petrylak, 2007) and CRPC normally represents a lethal form of PCa.

1.2.4 Prostate cancer diagnosis and prognosis

This section will describe the methods used for PCa diagnosis and assessing prognosis, including clinical symptoms, PSA testing, Gleason grades, clinical stages and biomarkers; along with the main diagnostic method used IHC staining.

1.2.4.1 Clinical symptoms

PCa is usually an asymptomatic (silent) disease as a result of its slow-growing nature (Hammerich *et al.*, 2008). However, some PCa patients show clinical symptoms such as frequent urination especially at night, bloody urine or semen, painful urination (Khan, 2011), urinary hesitancy (Hamilton and Sharp, 2004) and feeling of a not fully emptied bladder, as well as erectile dysfunction (Wlliamson, 2015). These symptoms, however, are not specific for PCa and may lead to misdiagnosis with other prostate disorders such as BPH (Khan, 2011), which represents a common hyperplastic disease in males (Hamilton and Sharp, 2004). Therefore, there are no specific clinical symptoms that can be used for PCa diagnosis.

1.2.4.2 Early diagnosis, screening and prostate-specific antigen

PSA is a protein produced by normal and malignant prostate epithelial cells (Adhyam and Gupta, 2012) and forms a part of semen contents. It is measured with a blood test and used clinically for PCa diagnosis and management (Epstein, 2010). The normal value for PSA in serum of men ranges from 0- 4 ng/ml (Dunn and Kazer, 2011) and these levels can increase in elderly men (Adhyam and Gupta, 2012) as well as in some pathological disorders of the prostate, such as bacterial prostatitis, BPH and PCa (Kawakami *et al.*, 2004; Lilja *et al.*, 2008; Dunn and Kazer, 2011). The increased PSA level seen in the serum of PCa patients is

thought to occur as a result of structural damage to the prostate epithelium, caused by events such as loss of the basal cell layer, ductal lumen architecture and epithelial cell polarity (Lilja *et al.*, 2008; Qu *et al.*, 2014).

The fact that PSA can be increased by bacterial prostatitis and BPH means that it is not a specific marker for PCa (Lilja *et al.*, 2008). There are also PCa patients who do not have increased PSA (Thompson *et al.*, 2004). For these reasons the United States prevention services task force doesn't recommend it to be used for routine screening, because of its low specificity in the PCa detection (Bensalah *et al.*, 2007) and routine screening is not currently carried out in the UK (Tikkinen *et al.*, 2018).

PSA tests are used for helping diagnose PCa in individual patients. These patients, who often present with the clinical symptoms described above, normally also have a rectal examination of the prostate. This is an additional diagnostic method and represents a more robust indicator of PCa than an abnormal level of PSA (Hamilton and Sharp, 2004). However, the detection of early stage PCa cases with an examination is hard.

In addition to being used for diagnosis, PSA testing is commonly used to monitor PCa patients after treatment (Kawakami *et al.*, 2004). PSA levels drop following surgery or radiotherapy, patients then have ongoing PSA monitoring as a subsequent increase in levels can be a sign of potential relapse.

Patients suspected to be at risk of PCa, based on clinical symptoms, a PSA test or a rectal examination normally then undergo a tissue biopsy, which is often taken with 18-gauge needles under transrectal ultrasound guidance (Heidenreich *et al.*, 2008). The biopsies allow confident diagnosis of PCa by analysing tissue architecture and staining for biomarkers with IHC. These two approaches are discussed below in sections 1.2.4.3 and 1.2.4.5.

1.2.4.3 The Gleason grading system

The tissue taken from a biopsy is normally stained using haematoxylin and eosin (H&E) to show the general structure of the tissues, such as the glands and stroma of the prostate (Varma *et al.*, 2002; Epstein, 2004) as well as to detect the pathological disorders such as BPH, PIN and PCa. (Dunn and Kazer, 2011). The tissue is also commonly stained with IHC to look at the expression of biomarkers (See section 1.2.4.6).

Having stained the tissue with H&E, the most common histopathological grading scheme which is used to measure the progression of PCa is the Gleason grading system (Kirby, 2012; Penney *et al.*, 2013). This system was developed by Donald F. Gleason in the 1960s and 1970s (Matoso and Epstein, 2016). It relies on an assessment of the histopathological architecture of the prostate and describes how much of the prostate tissue looks normal or abnormal. It is divided into five different Gleason grades (numbered from 1 to 5) (Gleason and Mellinger, 1974; Kirby, 2012) and the main histological changes are summarised below in (Figure 1.3 and Table 1.1). In a well-differentiated tumour, which represents the Gleason grade 1, the prostate glands are small, uniform in size, do not infiltrate, and the main characteristic features of tumour cells include small and uniform nuclei with pale cytoplasm (Delahunt *et al.*, 2012). At the other end of the scale, the architectural pattern of grade 5 is poorly differentiated glands with the tumour cells infiltrating the stroma of the prostate as cords, sheets, and nests. Between these are intermediate grades (named 2, 3, 4) (Epstein, 2010) (Figure 1.3).

PCa is a heterogeneous disease and can have two or more histopathological patterns in the same PCa sample, so a Gleason score which represents a combination or summation of the first and the second most predominant grades of PCa, is used (Matoso and Epstein, 2016). The first predominant (primary) grade in this system is given to describe the cells that form the largest area in cancer tissues, whereas, the second most predominant (secondary) grade is given to describe the cells of the next large area PCa (Matoso and Epstein, 2016). This scoring system ranges from 2 to 10 (Penney *et al.*, 2013) but clinically, most Gleason scores in PCa patients are 6 or more (Matoso and Epstein, 2016).

A more recent study has suggested a significant modification in this system (Matoso and Epstein, 2016). The new modified grading system consists of five grade groups named 1-5 and the first two Gleason scores from the original system are not applied (Matoso and Epstein, 2016). The lowest grade in this new system is called grade group 1 which represents a Gleason score ($\leq 3 + 3$) from the original one (Matoso and Epstein, 2016). Some of the specific morphological changes, which have been shown in Gleason score 6 from the original, are transferred into grade group 2 in the modified Gleason system to give a better prognostic value compared to the original system (Matoso and Epstein, 2016).

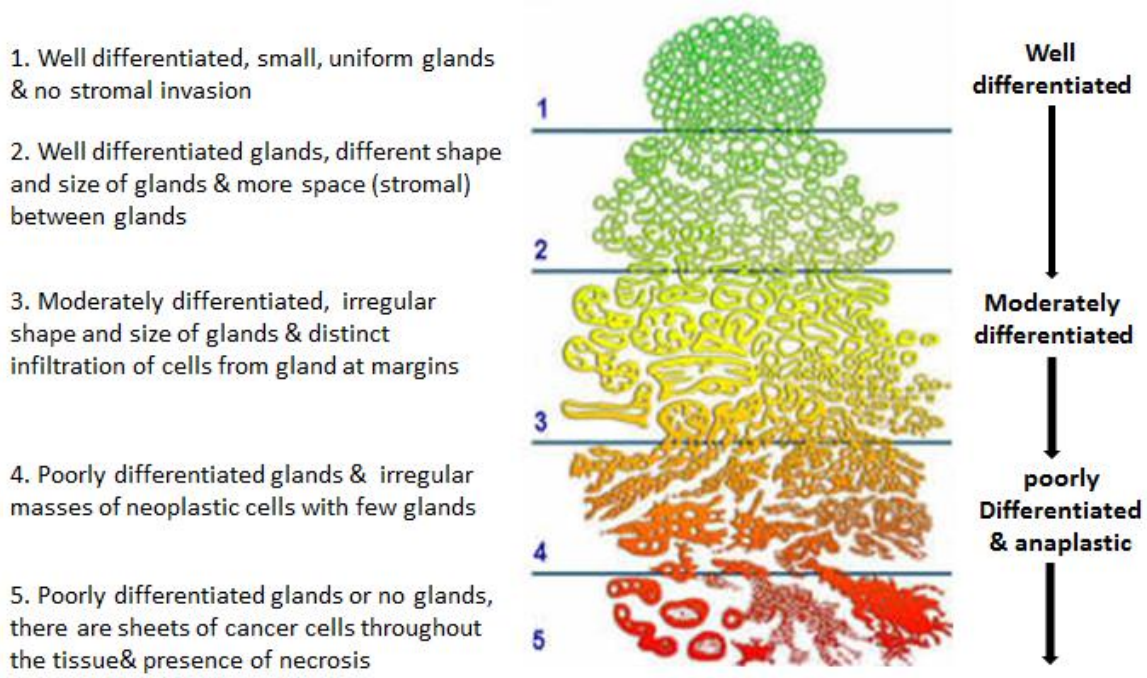

Figure 1. 3 Five Gleason grades of PCa. (grade 1) tumours consist of small, uniform glands with minimal nuclear changes. (grade 2) tumours have medium sized acini, still separated by stromal tissue. (grade 3) tumours show marked variation in glandular size, organization, and infiltrating stroma. (grade 4) tumours show marked cytological atypia an extensive infiltration. (grade 5) tumours are characterized by sheets of undifferentiated cancer cells. Adapted from (Tabesh *et al*., 2007).

The second modified grade group 2 represents a Gleason score 7 (3+4), whereas, the third one represents a Gleason score (4+3) from the original one (Matoso and Epstein, 2016). This is because patients with a Gleason score 7 (4+3) have a worse prognosis compared to those a Gleason score 7 (4+3) (Matoso and Epstein, 2016). The original Gleason score 8 (4+4) is called a grade group 4 and it has a worse prognosis value compared to the previous grading groups (Matoso and Epstein, 2016). Finally, Gleason score 9 (4+5 and 5+4) and Gleason score 10 (5+5) are both referred to as grade group 5 and represent the most histologically dedifferentiated tumour types in this system (Matoso and Epstein, 2016). The main histological changes in the new system are summarised in Table 1.1

The original and modified Gleason scoring systems are widely used to provide prognostic information and to determine the aggressiveness of the disease. For example, a high Gleason score is correlated with a higher risk of progression to a worse prognostic disease, elevated risk of metastasis, and reduced survival rate. In addition, the probability of tumour recurrence after radical prostatectomy is another phenomenon directly associated with a high percentage of grade 4 or 5 in the sample (Kirby, 2012). However, this system cannot always distinguish

between aggressive and indolent tumour types (Penney *et al.*, 2013). It also cannot predict those patients who will suffer from recurrence following primary therapy vs those that will remain in remission.

Table 1. 1 New prostate cancer grading system. Adapted from (Epstein et al., 2016)

New grading system	Gleason score	Histologic definition
Grade group 1	≤3 + 3 = 6	Only individual discrete well-formed glands
Grade group 2	3 + 4 = 7	Predominantly well-formed glands with lesser component of poorly formed/fused/cribriform glands
Grade group 3	4 + 3 = 7	Predominantly poorly formed/fused/cribriform glands with lesser component of poorly formed glands (a)
Grade group 4	8	Only poorly formed/fused/cribriform glands - Predominantly well-formed glands and a lesser component lacking glands (b). Predominantly lacking glands and a lesser component of well-formed glands
Grade group 5	9-10	Lack of gland formation (or with necrosis) with or without poorly formed/fused/cribriform glands (a)

Note : (a) For cases with >95 % poorly formed/fused/cribriform glands or lack of glands on a core or at radical prostatectomy, the component of <5 % well-formed glands is not factored into the grade. (b) Poorly formed/fused/cribriform glands can be a minor component.

1.2.4.4 Pathological staging system

There is another system used in PCa diagnosis and progression, in addition to a Gleason score, which is called the Tumor-Node-Metastasis system (TNM). This system was developed by the American joint committee on cancer / International Union against cancer (AJCC/ UICC) and depends on the size of the PCa and the amount of spreading that has occurred (Edge and Compton, 2010; Kirby, 2012).

The TNM system has three parameters, the amount of primary tumour progression in the prostate and other surrounding organs (T), the lymph node status (N) and presence or absence of metastasis (M) (Khan, 2011). The first parameter (T) in this system is based on the size of the tumour and amount of tumour spreading in the pelvic region. It is classified

into four subgroups (T1-4) (Figure 1.4) (Kirby, 2012) and a high score (T3 and T4) may suggest a greater chance of recurrence. The second parameter describes a spreading of the tumour to the lymph node and can be classified into two categories, including N0, which means that regional lymph nodes are not affected, and N1 which means the tumour has spread to the local lymph nodes (Epstein, 2010). Finally, the third parameter (M) refers to the metastatic status of diseases and can be divided into two categories M1 and M0, which means a tumour extends to other organs or not, respectively (Edge and Compton, 2010).

The benefit of this system is that it may be used in evaluating the prognosis of patients with PCa and can be used to assess the likely development of their cancer (Hammerich *et al.*, 2008), and may have a role as a guide for patient treatment planning (Cheng *et al.*, 2012). There are several modifications to the PCa staging system. This study used the 7th edition, from 2010 (Table 1.2) (Edge and Compton, 2010).

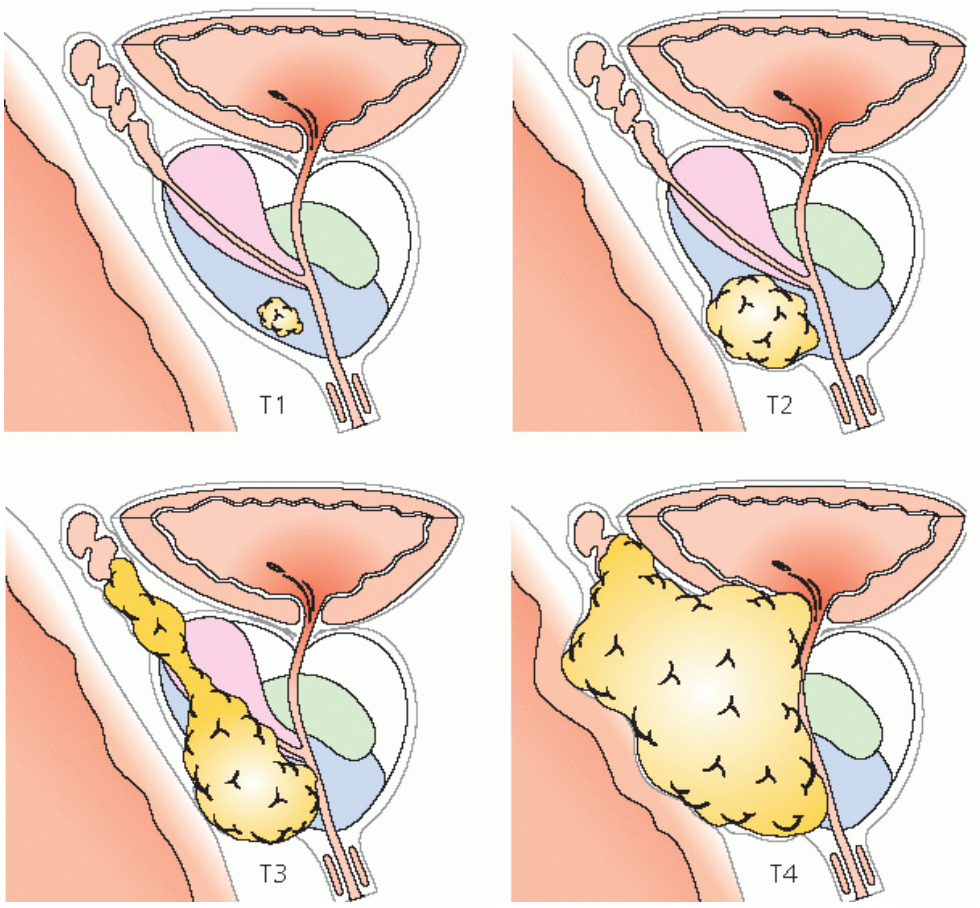

Figure 1. 4 Four stages of primary tumour progression, from T1 (incidental) to T4 (invasion of neighbouring organs). In T1, tumour size is small and is still inside of the prostate gland. Tumour size in T2 is larger than a T1 and involves more than one lobe. In stage T3, tumour involves prostatic capsule extends to another neighbouring organ, including seminal vesicles. Addition to seminal vesicle invasion, tumour invades other adjacent organs in the final stage (T4) such as bladder neck, external sphincter, rectum and elevator muscles and or pelvic wall. Adapted from (Kirby, 2012).

Table 1. 2 The TNM classification of prostate cancer (2010). Adapted from (Edge and Compton, 2010)

Primary tumours	
TX	A primary tumour cannot be assessed
T0	No evidence of a primary tumour
T1	Clinically inapparent tumour not palpable or visible by imaging T1a: Tumour incidental, histological finding in 5% or less of tissue resected. T1b: Tumour incidental, histological finding in more than 5% of tissue resected. T1c: Tumour identified by needle biopsy (for example, because of elevated PSA).
T2	Tumour confined within prostate*. T2a: Tumour involves one-half of one lobe or less. T2b: Tumour involves more than one-half of one lobe but not both lobes. T2c: Tumour involves both lobes
T3	Tumour extends through the prostate capsule ±. T3a: Extracapsular extension (unilateral or bilateral). T3b: Tumour invades seminal vesicle(s).
T4	A tumour is fixed or invades adjacent structures other than seminal vesicles, such as external sphincter, rectum, bladder, levator muscles, and/or pelvic wall.
Regional lymph nodes	
NX	Regional lymph nodes were not assessed.
N0	No regional lymph node metastasis.
N1	Regional lymph node metastasis.
Distant metastasis++	
MX	distant metastasis cannot be assessed

M0	No distant metastasis
M1	Distant metastasis ǂ. M1a: Non regional lymph node(s). M1b: bone(s). M1c: other site(s).
*: Tumour found in one or both lobes by needle biopsy, but not palpable or visible by imaging, is classified as T1c. ±: invasion into the prostatic apex or into (but not beyond) the prostatic capsule is not classified as T3 but as T2. ǂ: when more than one site of metastasis is present, the most advanced category should be used	

1.2.4.5 Biomarkers for the diagnosis of prostate cancer

A key step required for the accurate diagnosis of PCa is the demonstration of a loss of the basal cells (Goldstein *et al.*, 2010a). However, H&E staining used for the Gleason grading (discussed above), may not be able to reliably detect basal cells in the prostate glands (Varma *et al.*, 2002) and because of that, it is necessary to show the loss of staining for biomarkers that detect basal cells in prostate tissues. Biomarkers that are expressed in PCa, rather than being lost, are also used.

Previous study has identified several biomarkers that can detect the presence or absence of basal cells in normal and malignant prostate tissues (Brustmann, 2015). High molecular weight cytokeratin (HMWCK) represents the first differential diagnostic biomarker for PCa vs. BPH and is also called 34βE12 (Paner *et al.*, 2008). It is a specific marker that is expressed in the cytoplasm of basal cells. However, this marker is not used in isolation, but is used with other basal cell-associated markers (Paner *et al.*, 2008). P63 is another diagnostic biomarker for basal cells and represents a p53 homologous protein that is expressed in the nuclei of basal cells (Signoretti *et al.*, 2000). The specificity and sensitivity of p63 in detecting basal cells in benign tissues, especially for transurethral resection of the prostate (TURP) specimen, is higher than HMWCK (Shah *et al.*, 2002). This might be because p63 is a nuclear protein, whereas HMWCK is a cytoplasmic protein, and nonspecific staining for some cytoplasmic proteins can be significantly higher than nuclear ones (Paner *et al.*, 2008). However, a few PCa cases have a positive expression of

p63 (Tan *et al.*, 2015a) and the use of a basal cell-associated marker cocktail (a mixture of p63 and HMWCK) gives better results than using either marker alone (Shah *et al.*, 2004; Paner *et al.*, 2008).

A different approach to diagnose PCa is to use tumour cell biomarkers to show the presence of PCa tissue, rather than use basal cell biomarkers to show the loss of normal cells. Alpha methyl acyl coenzyme A racemase (AMACR) is a promising PCa cell biomarker (Luo *et al.*, 2002). It is an enzyme that plays an essential role in peroxisomal beta-oxidation of branched-chain fatty acid molecules (Luo *et al.*, 2002). Luo *et. al*. reported that AMACR is highly expressed in both PCa and HGPIN, whereas, the majority of NP tissues was a negative (Luo *et al.*, 2002).

Using a combination of AMACR and basal cell markers such as p63 and HMWCK can help the differentiation between malignant prostate cells and PIN. This combination is called a triple PIN cocktail (Paner *et al.*, 2008). A PIN cocktail has many advantages, including a greatly sensitive detection of PCa and reducing microscopic workload for pathologists (Tolonen *et al.*, 2011). However, Zhou *et al* showed that 18 % of PCa previously diagnosed by H&E and with a negative basal cell marker, had negative AMACR staining. These findings would suggest that accurate diagnosis of PCa requires a combination of biomarkers used together and no single marker can be used to distinguish PIN from PCa (Zhou *et al.*, 2004).

1.2.4.6 IHC in prostate cancer diagnosis

IHC is a method that is routinely used to stain for PCa biomarkers in clinical pathology laboratories. It is also extensively used in this thesis. It is a technique that can be used to stain components of cells and tissues in cancerous and non-cancerous conditions (Oliver and Jamur, 2010), including basal cells in prostate tissues (Brustmann, 2015). The IHC principle depends on antigen-antibody interactions (Oliver and Jamur, 2010). The antigens that are normally present in cells or tissues (Oliver and Jamur, 2010) can be detected by using antibodies which can bind to these antigens. The site of antibody binding can be identified by using a directly labelled antibody or using a secondary labelling method (Figure 1.5).

Direct IHC is is a quick method that depends on a direct interaction between an enzyme-labelled primary antibody and antigens in tissues (Ramos-Vara, 2017) (Figure 1.5 A). The sensitivity of this method is weak compared to the other IHC types due to lack of signal

amplification and can be only used for detecting highly expressed antigens (Boenisch, 2001). Another weakness is that a label for each primary antibody is necessary (Ramos-Vara, 2017). Indirect IHC has been developed to avoid these problems. During indirect IHC a tissue antigen interacts directly with an unconjugated primary antibody which is then bound by an enzyme- labelled secondary (Magaki *et al.*, 2019) (Figure 1.5 B). This method is more sensitive than the direct one because of a number of labelled secondary antibodies can bind to different epitopes, allowing for amplification steps increase signal intensity (Boenisch, 2001). However, the use of a secondary antibody that needs additional blocking steps and controls. There is another indirect IHC method called EnVision in which tissue antigens can bind directly to a primary antibody which is followed by a spine polymer molecule which contains an average of 10 molecules of secondary antibodies and 70 molecules of peroxidase (Boenisch, 2001) (Figure 1.5 C). This method is very sensitive compared to the previous methods due to increased amplification and it was the IHC method used in this study.

The antigen-antibody interaction in all these IHC methods is normally visualised by using a chromogenic substrate which uses enzymatic labels to generate an insoluble, coloured deposit at the site of interaction. The most common system depends on using either diaminobenzidine/ horseradish peroxidase (DAB/HRP) or alkaline phosphatase/ Fast Red (AP/FR)which can give colour for a positive interaction (Figure 1.5).

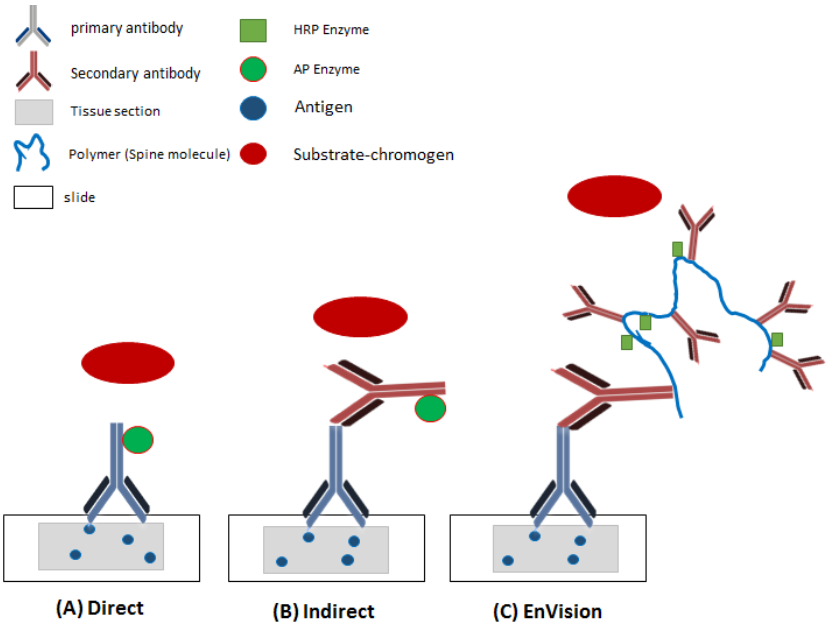

Figure 1. 5 Principle of immunohistochemistry. (A) Direct IHC: An enzyme- labelled primary antibody reacts directly with tissue antigen. (B) Indirect two-step IHC: An antigen binds directly with a primary antibody, which binds with an enzyme- labelled secondary antibody. (C) Indirect EnVision IHC: An antigen binds directly with a primary antibody which is followed by a spine polymer molecule which contains an average of 10 molecules of secondary antibodies and 70 molecules of peroxidase). The reaction is visualised by using a DAB substrate- chromogen solution. Adapted from (Boenisch, 2001).

1.2.5 Molecular changes in prostate cancer

In this section, the most common genetic alterations and signalling aberrations that appear during the transition from NP to PIA, PIN, carcinoma and finally to CRPC (Figure 1.7) will be discussed.

1.2.5.1 Glutathione S -transferase π

Glutathione S -transferase π (GSTP1) is a carcinogen-detoxifying enzyme that is normally expressed in basal cells of the prostate gland and is also found to be highly expressed in luminal and basal cells of PIA (De Marzo *et al*., 1999). In contrast, 90% of PCa tissues show non-significant staining for GSTP1 (De Marzo *et al*., 1999) (Figure 1.7).

Functionally, GSTP1 plays a role in protecting cells from carcinogenic factors as a result of its role as a detoxifying enzyme which is able to catalyse the conjugation of glutathione with harmful, electrophilic molecules that are endogenously or exogenously produced (Porkka and Visakorpi, 2004). The presence of GSTP1 in NP tissues may play an important role in protecting cells from oxidative or electrophilic DNA damage (De Marzo *et al*., 1999). A previous study reported that GSTP1 CpG island hypermethylation is frequently detected in the majority of PCa cases (>90%) as well as in DNA of 69% of PIN and only 6% of PIA (Nakayama *et al.*, 2003), resulting in downregulation of GSTP1 protein level. In contrast, hypermethylation has not been detected in DNA of all non-malignant prostate cases, such as normal and BPH (Nakayama *et al.*, 2003). The data suggest that methylation of GSTP1 may control its expression and GSTP1 hypermethylation represents an early step in PCa progression.

1.2.5.2 NKX3.1

Homeobox NKX3.1 is a transcription factor and is thought to be a prostatic tumour suppressor. It appears to represent one of the earliest markers identified for the prostate epithelium during embryogenesis (Bhatia-Gaur *et al*., 1999). Its expression depends on androgen signalling (Tan *et al.*, 2012). It is expressed in benign tissues of the prostate (Bhatia-Gaur *et al.*, 1999; Bowen *et al.*, 2000; Gurel *et al.*, 2010), but its expression levels in PCa cases are decreased and this reduction may represent one of the most critical events for PCa initiation (Abate-Shen *et al*., 2008). Bowen *et al* have also found that NKX3.1 was expressed in 95% of NP, but its expression has been lost in 20% of HGPIN, 6% of early

stage PCa (T1) and 22% advanced stage of PCa (T3/4) (Bowen *et al.*, 2000). In addition, it was also found to be lost in 35 % of CRPC and 78% of advanced stage PCa (Bowen *et al.*, 2000), suggesting that the loss of NKX3.1 expression is an early event in PCa formation and is strongly associated with CRPC and advanced PCa stages (Figure 1.7).

The gene encoding NKX3.1 is localised at chromosome 8p21 (Gurel *et al*., 2010), which is commonly deleted in PIN lesions (Irshad *et al.*, 2013). A study on mice has also found that a single NKX3.1 allele deletion is capable of generating PIN lesions (Bhatia-Gaur *et al.*, 1999). Another study showed that the NKX3.1 locus is also frequently deleted in PCa (Voeller *et al.*, 1997), but no mutations were found in the remaining allele of NKX3.1 (Voeller *et al.*, 1997), suggesting that haploinsufficiency (i.e. inactivation of only one allele) could be the tumour suppressor mechanism for NKX3.1 in PCa (Porkka and Visakorpi, 2004).

Functional studies using mouse models have shown that NKX3.1 has a critical regulatory role in the proliferation of prostate epithelial cells and differentiation of bulbourethral glands. For example, it has been found that NKX3.1 is required for prostatic epithelial differentiation in mice and plays a regulatory action in NP development, whereas its mutation in mice may lead to having a defect in ductal morphogenesis and production of secretory proteins (Bhatia-Gaur *et al*., 1999). Another study has suggested that NKX3.1 deficiency in mutant mice may lead to an increase in the oxidative damage to DNA and protein as well as decreased expression of oxidative enzymes, suggesting that NKX3.1 protein may have a role in regulating the response to oxidative stress which can play a significant role in tumour initiation (Ouyang *et al.*, 2005). This study also reported that NKX3.1 plays a role in maintaining the integrity of prostate epithelial layers through regulation of the expression of genes which protect DNA from oxidative damage (Ouyang *et al.*, 2005). Taken together, this evidence suggests that NKX3.1 is downregulated at an early stage of prostate tumorigenesis, that more complete loss of NKX3.1 might be associated with PCa relapse and metastatic diseases, and that reduction of its function contributes to PCa formation and progression.

1.2.5.3 Myc

The Myc proto-oncogene is a transcription factor located on chromosome 8q24 (Yoshida, 2018) which is frequently altered in human carcinogenesis, including PCa (Koh *et al.*, 2010;

Yoshida, 2018). Previous studies have reported that Myc genes are overexpressed in PIN lesions (Gurel *et al*., 2008; Iwata *et al*., 2010), as well as in prostate tissues with malignancies compared to NP or BPH (Buttyan *et al.*, 1987; Fleming *et al.*, 1986; Gurel *et al.*, 2008) (Figure 1.7).

Functionally, it has been found that Myc could play a role in regulating cell proliferation, metabolism, protein synthesis function and SC renewal (Gurel *et al.*, 2008) as well as cancer initiation (Koh *et al.*, 2010). Several mechanisms have been suggested for Myc to induce pre-neoplastic or neoplastic lesions such as PCa. First, a study has found that increased Myc plays a vital role in transforming normal mice prostate cells to PIN lesions. Knockdown of Myc by siRNA increased NKX3.1 protein and mRNA levels in human PCa cell lines (Iwata *et al.*, 2010), suggesting Myc induces PIN through repression of NKX3.1. In addition, Ellwood-Yen *et al* found that overexpression of Myc in transgenic mice induces a rapid formation of PIN and then invasive adenocarcinoma (Ellwood-Yen *et al.*, 2003). There is a significant association between PI3K-pathway alterations and Myc multicopy gain in PCa metastases and there is a cooperation between Myc and AKT to accelerate the progression of PIN to invasion in a murine PCa model (Clegg *et al.*, 2011). In addition, it has been found that Myc can bind with its coactivator Max (Myc associated factor X), to form a Myc- Max complex which acts to activates transcription of a large cohort of target genes and promote cell growth and proliferation (Weinberg, 2007). Taken together, Myc could play an essential role in PCa formation and progression through its collaboration with other driver pathways.

1.2.5.4 Phosphatase and tensin homolog

Phosphatase and tensin homolog (PTEN) is a tumour suppressor gene which is commonly mutated or deleted in different types of human cancer, including PCa (Jamaspishvili *et al*., 2018; Salmena *et al*., 2008). This gene is located on chromosome 10q23, a region that is commonly altered in PCa (DeMarzo *et al*., 2003). PTEN expression was observed in 92% of PIN (24/26), and 86% of PCa (50/51) using IHC (Fenic *et al*., 2004). This reduction was also positively associated with a risk of biochemical recurrence (Chaux *et al.*, 2012) (Figure 1.7). This evidence suggests that loss of PTEN in the prostate gland drives tumorigenesis and is associated with the emergence of recurrence (Shen and Abate-Shen, 2007; Irshad and Abate-Shen, 2013). A previous study has reported decreased PTEN expression in a Sall-like 4 (Sall4) B transgenic mice compared to wild-type control (Lu *et al.*, 2009), suggesting

PTEN level can be regulated by Sall4 and increased Sall4 level represses PTEN followed by activation of PI3K/AKT pathway and enhanced PCa formation.

Functionally, PTEN is a lipid phosphatase that converts phosphatidylinositol 3, 4, 5 triphosphates (PIP3) to Phosphatidylinositol 4,5 phosphate (PIP2), thereby directly inactivating the PI3K/AKT pathway that plays a role in promoting cell survival and inhibiting apoptosis (Bertrand *et al.*, 2014; Leevers *et al*., 1999; Porkka and Visakorpi, 2004). Increased proliferation and decreased sensitivity to apoptosis have also been found in mutant PTEN mice (Stambolic *et al.*, 1998).

In addition to its role in promoting proliferation and apoptosis, PTEN in the nucleus plays an important role in chromosome stability, DNA repair, cell cycle arrest and cellular stability (Planchon *et al.*, 2008). In addition, other studies have reported inactivation of PTEN cooperated with other mutated genes to promote progression of HGPIN to invasive adenocarcinoma, including loss of NKX3.1 (Kim *et al.*, 2002), increased c-Myc (Kaur and Cole, 2013; Kim *et al.*, 2009) and Transmembrane protease, serine 2- ETS- regulated gene (TMPRSS-ERG) fusion (Carver *et al.*, 2009). Taken together, PTEN loss is one of the most important mechanisms for PCa formation and progression through its cooperation with other genes.

1.2.5.5 TMPRSS2-ERG gene fusion

Another key alteration that has been identified in PCa is increased expression of E26 transformation-specific (ETS) transcription factor family target genes, including the ETS proteins ERGs (Smith *et al*., 2012). ERG genes are located on the same chromosome, 21q22.2, as TMPRSS2 (Demichelis and Rubin, 2007) and TMPRSS2-ERG gene fusion represents one of the most common genetic alteration identified in PIN (19%) and more than a half of PCa patients (Perner *et al.*, 2007; Squire, 2009; Hagglof *et al*., 2014; Yang *et al*., 2016). It is also associated significantly with biochemical recurrence (Nam *et al*., 2007). This fusion, however, has not been detected in NP or PIA tissues (Demichelis and Rubin, 2007). The evidence suggests that TMPRSS2-ERG fusion is an indicator of PCa formation, aggressiveness and relapse (Figure 1.7).

There are two possible mechanisms that have been proposed to explain the fusion between TMPRSS2 and ERG genes. First, a large genetic area of chromosome 21, which is located between these two genes is deleted (Hossain and Bostwick, 2013). Second, a translocation of these two genes may occur (Hossain and Bostwick, 2013) (Figure 1.6).

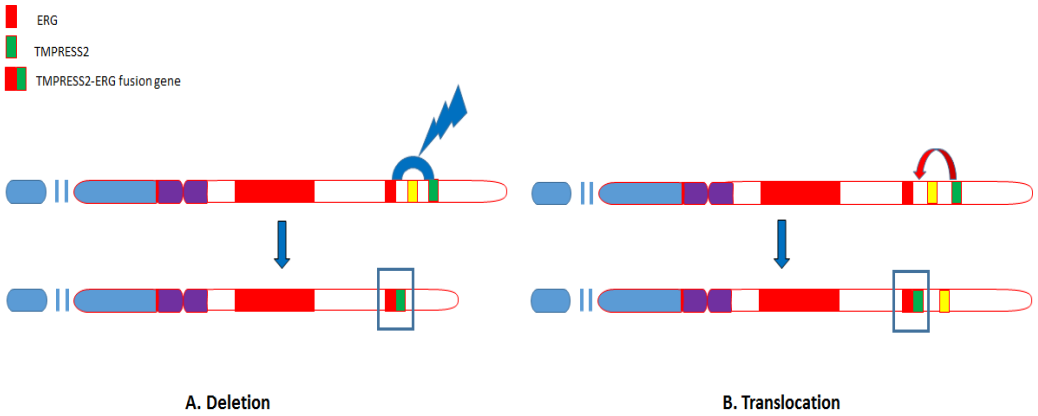

Figure 1. 6 Mechanisms of TMPRSS2–ERG gene fusions at chromosome 21. (A) Deletion: A large part of the chromosome between these genes (ERG and TMPRSS2) is deleted. (B) Translocation: TMPRSS2 gene is translocated to be fused with ERG. Adapted from (Hossain and Bostwick, 2013).

Previous studies have reported mechanisms that may explain the possible role of this fusion in PCa. TMPRSS2 is regulated by AR activity which means that after fusion TMPRSS2 promoter elements drive ERG expression in PCa (Tomlins *et al.*, 2005). Another study has found that AR signalling might be disrupted by ERG through its role in inhibiting AR expression and interacting to and inhibiting AR downstream targets at gene-specific loci, and increased EGR, which may occur as a result of this fusion in PCa may lead to increased PCa cell invasion and growth in the existence or absence of androgens, independent of AR (Yu *et al.*, 2010). This fusion might disrupt also the capability of cells to differentiate into NP cells as well as play an essential role in PCa development (Yu *et al.*, 2010). This evidence suggests that TMPRSS2–ERG fusion may play a role in CRPC development through its inhibitory role to AR signalling. Finally, transcriptional downstream checkpoint genes can be induced by a combination of ERG and PTEN loss, promoting cell proliferation, DNA repair and survival (Squire, 2009). ERG and PTEN may, therefore, deliver selective benefit to permit PIN lesions to progress to PCa and ultimately CRPC (Squire, 2009).

1.2.5.6 Phosphoprotein 53

Phosphoprotein 53 (p53) is a tumour suppressor gene that resides on the short arm of chromosome 17 (17q13.1) (Chappell *et al.*, 2012; Heidenberg *et al.*, 1996). It has been observed that p53 is frequently mutated in cancer (Hainaut *et al.*, 1998), and its mutation is

found to be associated with poor outcomes following hormone therapy in advanced prostate (Chappell *et al.*, 2012) and risk of recurrence (Hamid *et al*., 2018) (Figure 1.7).

Several studies have examined the expression of p53 in normal and malignant prostate, including recurrence and non- recurrence. Some of these have described an increase in P53 expression. For example, a previous study found increased p53 in PCa compared to BPH (Kuczyk *et al.*, 1998). Other studies looking at p53 protein levels found that increased p53 is also significantly associated with PCa Gleason grade and clinical stage (Aprikian *et al.*, 1994) as well as recurrence (Aprikian *et al.*, 1994; Brewster *et al.*, 1999; Porkka and Visakorpi, 2004). In addition, there is a negative association between increased p53 expression and long term survival following radical prostatectomy (Kuczyk *et al.*, 1998).

In addition to increased expression of p53, it is well established that it can be reduced in PCa. This reduction occurs frequently as a result of the loss of one allele, coupled with inactivation of the second allele by an intragenic mutation (Hruban *et al.*, 1999) and p53 is mutated in about 35 % of advanced PCa cases (Ecke *et al.*, 2010). In addition, a previous study on PTEN null transgenic model found that inactivation of p53 may lead to progress of a PIN lesion to invasive carcinoma (Chen *et al.*, 2005). Navone, *et al* have demonstrated that p53 gene mutation is observed in late-stage PCa and metastasis and is also significantly associated with loss cell differentiation and the transition from androgen-dependent to androgen-independent growth (Navone *et al.*, 1993). In contrast, this alteration was not observed previously in BPH or PIN (Navone *et al.*, 1993).

Exposure of cells to a variety of cell physiologic stress such as UV radiation, ionizing radiation, hypoxia, oncogene signalling and transcription blockage can result in increased p53 levels that can induce a number of processes such as cell cycle arrest, mobilisation of DNA repair proteins, block of angiogenesis and triggering of apoptosis (Weinberg, 2007). p53 plays an essential role in regulating cell proliferation and apoptosis and loss of its function may have a strong effect on cell cycle regulation (Hruban *et al.*, 1999). Decreased p53 transcriptional activity is negatively associated with PCa colony growth (Chappell *et al.*, 2012), suggesting it could play a vital role in PCa progression. The evidence shows that p53 is a critical element in regulating a cells ability to control the cell cycle and could play an essential role in PIN transition to carcinoma, as well as for tumour progression to metastasis or relapse.

1.2.5.7 Androgen receptor

It is known that androgens and their receptor have an essential role in the development of the prostate (Shen and Abate-Shen, 2010). Androgens are also necessary for PCa growth and survival as a result of its key role as the main regulator of cell proliferation and apoptosis ratio in prostate cells (Tan *et al.*, 2015b) (Figure 1.7).

The main circulating androgen in the human body is testosterone, which is synthesised by the testis, but there is also another source of androgen which comes from the adrenal gland (Tan *et al*., 2015b; Zhu and Garcia, 2013). Inside prostate cells, the majority of testosterone becomes converted by 5α-reductase enzyme to the more active hormone dihydrotestosterone (DHT), which has a strong affinity for the AR (Feldman and Feldman, 2001). AR is a nuclear receptor that is normally bound to proteins, including heat shock protein (HSP) in a conformation that stops DNA binding and receptor phosphorylation (Feldman and Feldman, 2001). However, DHT binding to the AR can induce changes in AR that play a role in promoting the dissociation of HSP from AR (Tan *et al.,* 2015b). After these changes, the complex translocates to the nucleus and then binds to androgen response elements (AREs) in the promoter regions of target genes, including PSA and TMPRSS2 (Tan *et al.,* 2015b). In addition, various coactivators (such as ARA70), corepressors, TATA box binding protein (TBP) and transcription factor IIF (TFIIF) can be recruited by AR at promoter regions (Feldman and Feldman, 2001; Tan *et al.*, 2015b). This activated complex can lead to induction of biological responses including growth, survival and the production of PSA (Feldman and Feldman, 2001; Tan *et al.,* 2015b).

Mutant AR has been observed rarely in untreated PCa (Culig *et al.,* 2001). In contrast, other studies found that frequent mutation for AR was observed in CRPC (Gaddipati *et al.,* 1994). In addition, a quarter of PCa patients who are treated with anti-androgen drugs have mutant AR (Wallen *et al.*, 1999) that is thought to help promote the transition to CRPC. There are a number of mechanisms that explain how these mutations in AR, and other changes, can cause CRPC which are discussed below.

The first possible mechanism for triggering the transition to CRPC is called the hypersensitive pathway. The AR gains sensitivity, which may be achieved through genetic alteration or amplification of AR or increased 5α reductase enzyme levels, so it responds to the very low concentration of androgens that may be found after castration or medical

androgen blockage (Dutt and Gao, 2009). The second mechanism is called the promiscuous pathway, in which mutant AR can interact with alternative ligands other than DHT, including oestrogen, progesterone and glucocorticoids and /or AR antagonists, resulting in PCa growth due to reactivation of the AR (Buchanan *et al.*, 2001). The outlaw pathway (alternatively known a ligand-independent pathway) is the third possible mechanism driving CRPC, in which a number of growth factors, including fibroblast growth factor, Insulin-like growth factor1, vascular endothelial growth factor; transforming growth factor-β, epidermal growth factor, and keratinocyte growth factor or tyrosine kinase such as HER-2/neu can bind and activate AR when androgen is absent or present at sub-physiological concentration (Culig *et al.*, 1995; Zhu and Kyprianou, 2008; Dutt and Gao, 2009), resulting PCa growth. The fourth mechanism which is called the AR-independent bypass pathway supports proliferation and survival of CRPC by a couple of proteins, including anti-apoptotic protein Bcl-2 (B cell lymphoma 2) and GR (Glucocorticoid receptor) (Hoang *et al.*, 2016). A previous study, using the LNCap prostate tumour model found decreased expression level of Bcl-2 delayed the growth of CRPC (Gleave *et al.*, 1999), suggesting increased Bcl-2 levels in cancer cells may play a role in the transition to CRPC. In addition, it has been demonstrated that GR is upregulated in CRPC and could play an important role as a substitute for AR in binding with androgen response elements and is necessary for cancer cells survival under certain circumstances (Arora *et al.*, 2013). A very recent study reported that immunostaining of a forkhead transcription factor (FoxA 2), whose expression is normally driven by AR-responsive promoters, is detected and localised in CRPC cells that show negative staining for AR, using IHC and IF, respectively (Connelly *et al.*, 2018). This study reported that regardless of the presence of androgens, FoxA 2 cells were fast growing compared to control cells. This suggested FoxA 2 may play a role in activating CRPC (Connelly *et al.*, 2018).

Overall, AR plays an important role in the development of both normal and malignant prostate through its regulatory role in cellular events, including proliferation, invasion, and differentiation. In addition, changes in AR signalling play a pivotal role in driving the progression from PCa to CRPC through many different mechanisms, including AR amplification, AR mutation, ligand-independent AR activation, bypass AR signalling mechanisms as well as activation of AR by FoxA 2.

1.2.5.8 AR coactivators and regulators

Previous studies have found many AR coactivators linked to PCa. However, in this section, a couple of AR co-activators which are frequently altered in PCa, including CRPC are described (Figure 1.7). For example, a recent *in vivo* study done by Blattner *et al.* has reported that a mutant Speckle-Type POZ Protein (SPOP) can drive PCa through its a role in activating PI3K/mTOR pathway as well as upregulation of AR network of AR-associated transcription factors and co-activators (Blattner *et al.*, 2017). SPOP mutations have been reported in about 10% of PCa (Barbieri *et al.*, 2012; Blattner *et al.*, 2014; Blattner *et al.*, 2017), which are ETS- fusion negative (Barbieri *et al.*, 2012). This co-activator can play a role in targeting AR for degradation, however, this role is lost in the event of a mutation (An *et al*., 2014). In addition to SPOP, another AR coactivator called steroid receptor coactivator-3 (SRC-3) has been found to be increased in tumours and has an essential role in androgen-dependent and androgen independent cell proliferation in PCa cell lines *in vitro* and xenografts *in vivo* as a result of its coordinate role with AR in promoting cell cycle gene expression (Zou *et al.*, 2006). SRC-2 is a third AR coactivator that is found to be amplified frequently in PCa, and amplification of the chromosomal region of 8q, the region of SRC-2 gene, is shown in 20% in primary and 63% in metastatic prostate tumours (Taylor *et al.*, 2010a).

1.2.5.9 Summary

PIA is an atrophic lesion in prostate architecture and is thought to be a precursor source of PCa formation through PIN, which represents the intermediate state between cancerous and noncancerous prostate. A couple of histological and genetic changes have been shown to occur during the transition from P1A to PIN, including a partial loss of both basal cells and basement membrane, a reduction of GSTP1, NKX3.1and PTEN expression as well as increased cell proliferation and inhibition of the apoptotic rate (Figure 1.7).

A number of significant changes have been identified during the transformation from PIN to carcinoma, including histological and molecular changes. The main histological changes are the total loss of basal cells and the basement membrane. Genetic alterations been reported in this transition include the loss of genes, such as NKX3.1, PTEN and p53, a high rate of TMPRSS2-ETS gene fusion, increased amplification and expression of Myc. These changes

play a vital role in regulating of PI3K-AKT, AR and other pathways important for PCa progression.

Finally, the transition to CRPC is driven by genetic alterations that alter AR signalling through its amplification or mutation or via AR-independent mechanisms, such as ligand-independent or bypass AR signalling mechanisms (Figure 1.7).

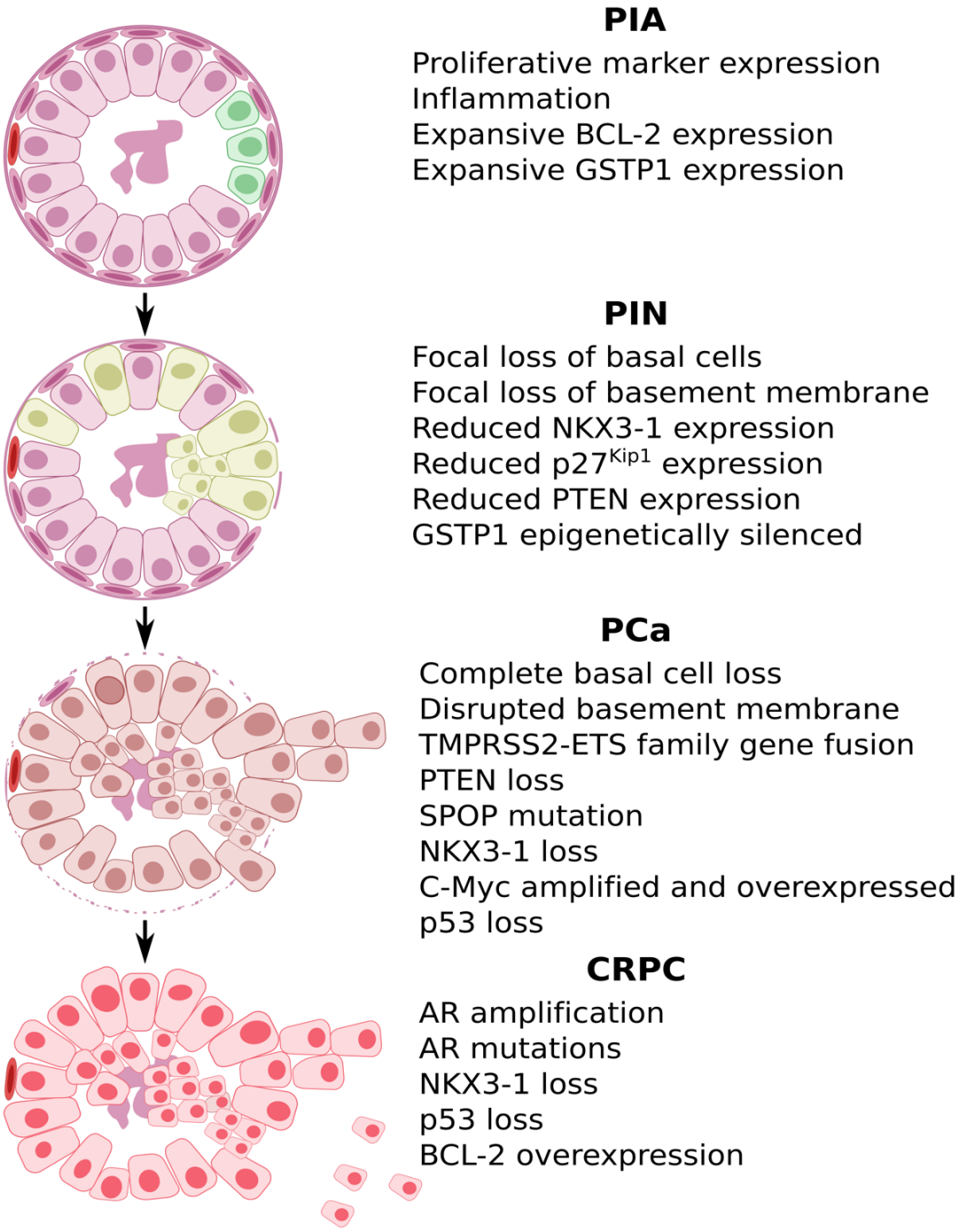

Figure 1. 7 Key oncogenic events in the carcinogenesis of the prostate. The key molecular changes such as gene mutations, losses and rearrangements, are shown next to a visual representation of each stage of prostate carcinogenesis. This model has the main stages of prostate carcinogenesis, including PIA (green cells), PIN, (yellow cells); PCa (brown cells); and CRPC (red cells). Adapted from (Sharpe, 2016).

1.3 Stem cells

The work described above has begun to describe the molecular basis of PCa initiation and progression, but there are many avenues that remain to be investigated. One exciting area of current research is the role that SCs play in PCa initiation and progression.

The term SCs has come to be used to refer to immature cells normally found in embryos and in small populations in both normal and malignant tissues, including prostate (Majumder, 2009; Tu and Lin, 2012). Previous work has found the main properties for these cells, include self-renewal and pluripotency (Isaacs, 2008; Tu and Lin, 2012). Self-renewal means that SCs have the ability to divide and create new SCs with the same development and replicative potential, while pluripotency means these cells are able to differentiate into specialised cell types (Lobo *et al.*, 2007).

SCs are often found in a cellular location called a niche, which probably offers a microenvironment necessary for the maintenance of the SC properties described above via a combination of intracellular and intercellular signalling (Lang *et al.*, 2009a; Prajapati *et al.*, 2013). Specific factors provided by the niche can include growth factors, cytokines, chemokines, and adhesive molecules which can regulate the balance between proliferation, differentiation, and quiescence in SC populations (Whetton and Graham, 1999; Spradling *et al.*, 2001).

SCs can be divided into two broad types, embryonic (ESC) and adult SCs (Tuch, 2006). ESCs, which are derived from the inner cell mass (ICM) of a growing mouse or human blastocyst (Majumder, 2009; Findikli, 2012) have the potential to become all cell types of the body because they are pluripotent (Tuch, 2006). During embryogenesis, tissue-restricted SCs, called adult SCs are created (Isaacs, 2008). Adult SCs, alternatively known as somatic or non-embryonic SCs, are found in many organs including the skin, intestine, bone marrow and the prostate. Adult SCs have a limited potential to differentiate and are normally restricted to forming the cell types of the tissue of their origin (Tuch, 2006). Interestingly, adult SCs have also been described in tumour tissue, including PCa tissue. The next section will focus on prostate SCs (PSCs) and prostate CSCs (PCSCs)

1.3.1 Prostate stem cells

The study of PSCs is at a fairly early stage and two main models for their location and properties have been described (Figure 1.8).

The first model, which represents the best-accepted description of PSCs suggests that PSCs are located in the prostate basal layer and can generate intermediate cells called transient amplifying (TA) (immature cells) which subsequently differentiate into the mature and fully differentiated luminal cells (Isaacs and Coffey, 1989; Takao and Tsujimura, 2008). This model has been confirmed by previous studies which found that there is a cell population located on the basal layer of the prostate gland of both human and mouse that possessed self-renewal capabilities and can generate both basal and luminal cells (Goldstein *et al.*, 2010b; Taylor *et al.*, 2010b). For example, previous work done by Goldstein *et al.* has separated the luminal cells (Trop2+/CD49f−) from the basal (Trop2+/CD49f+) cells in digests of benign human prostate tissues and these cells (luminal and basal) were then injected subcutaneously into immunodeficient mice (Goldstein *et al.*, 2010b). The basal cell population give rise to prostate-like structures containing both basal and luminal cells, whereas the luminal population did not grow (Goldstein *et al.*, 2010b). In addition, it has been found that basal cells have the ability for survival following androgen ablation (Wang *et al*., 2013). Another study on BPH suggested that basal cells might be able to give rise to luminal cells because actively dividing cells were predominantly localized to the basal compartment (Dermer, 1978). In addition, several SC characteristics have been found in the basal cells, including their relatively undifferentiated state, high proliferative capacity, protection from apoptosis, and a long life span (Bonkhoff *et al.*, 1994; De Marzo *et al.*, 1998; Foster *et al.*, 2002; Wang *et al.*, 2013). The TA cells are able to express both luminal and basal cell markers such as CK5, CK8, CK14, CK18, AR, and PSA (Bonkhoff *et al.*, 1994; Bonkhoff and Remberger, 1996; Xue *et al.*, 1998).

The second model suggests that both prostate gland luminal and basal layers have a small population of PSCs which subsequently differentiate into mature basal and luminal cells, respectively (Takao and Tsujimura, 2008) (Figure1.8). The evidence for this model is that the majority of cells present in PCa are luminal-like cells and unlike normal luminal cells, these cells are not fully differentiated cells (Jaworska *et al.*, 2015), suggesting that PCa most often arises from luminal, undifferentiated cells and these cells could be SCs or CSCs. It has been reported that long-term BrdU label (^{3}H-thymidine or 5-bromo-2-deoxyuridine)

retention experiments label cells of both basal and luminal mouse prostate, even after many cycles of androgen ablation (Tsujimura *et al.*, 2002), suggesting that PSCs may be not restricted to the basal compartment.

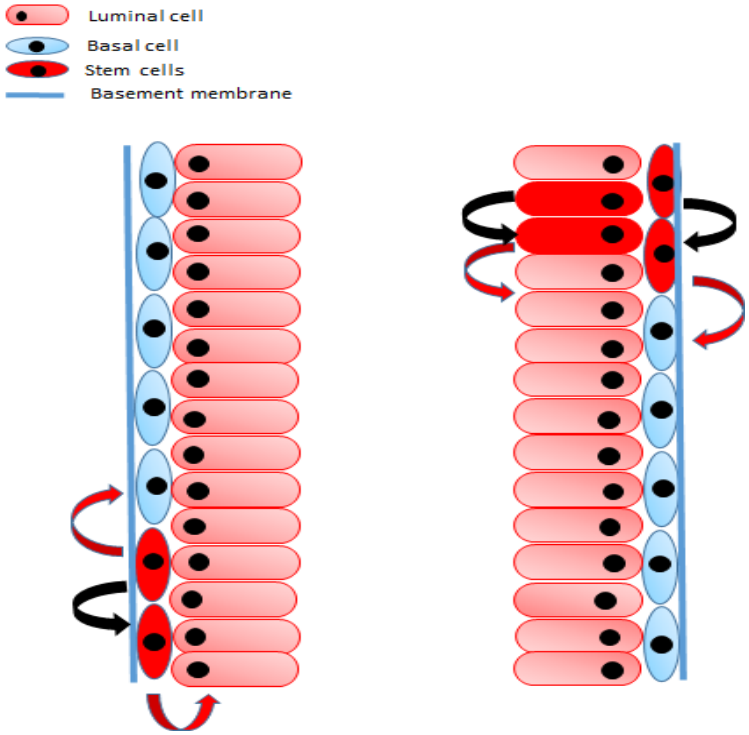

Figure 1. 8 The possible models of PSCs. The first model suggests that PCSc occur in the basal cell layer and generate TA cells to be able to produce fully differentiated luminal cells (left side). In contrast, the second model is thought that both the basal and luminal layers have a small population of stem cells which are able to differentiate into mature basal or luminal cells, respectively (right side). Adapted from (Takao and Tsujimura, 2008).

1.3.2 Prostate cancer stem cells

PCa consists of a heterogeneous population of cells with varying degrees of tumorigenic potential, and only a subset of cancer cells, which are called PCSCs can initiate and propagate a tumour. CSCs, including PCSCs, have been defined according to the American Association for Cancer Research (AACR) as "a cell within a tumour that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise a tumour"(Clarke *et al.*, 2006). They possess most of the properties of adult SCs, they are long-lived, slow cycling, self-renewing cells that can differentiate (Jaworska *et al.*, 2015).

The existence of PCSCs has been examined and as with many cancers is still open for discussion. However, several approaches have been used to identify and isolate CSCs such as side populations (SP) (Patrawala *et al.*, 2005), sphere formation (Zhang *et al.*, 2012) and surface markers (Hurt *et al.*, 2008). First, a previous study done by Patrawala *et al.* has

analysed SP in several PCa cell lines, including Du145, LAPC-4, and LAPC-9 and found that LAPC-9 xenograft tumours contained detectable SP cells whereas the other cell lines did not (Patrawala *et al.*, 2005). This work found that LAPC-9 cells have a higher tumorigenicity than those cell lines without SP, with as few as 100 SP cells giving rise to tumors (25% incidence), whereas 300,000 non-SP cells were wanted to create a tumour (Patrawala *et al.*, 2005), suggesting the SP cells had SCs properties (Patrawala *et al.*, 2005). Second, a sphere formation assay has been used for the enrichment of populations of CSCs from PCa cell lines (Zhang *et al.*, 2012) or PCa tissues (Castellon *et al.*, 2012). Zhang and colleagues reported less than 5% of PCa cells, which are cultured in non-adherent suspension condition with serum-free medium, are capable of forming prostatosphere in culture suspensions and these small population cells have a high self-renewal and tumour initiation properties, using LNCaP, 22RV1, DU145 and PC-3 cell lines (Zhang *et al.*, 2012). A small population of cells obtained from prostatospheres showed SC marker expression such as CD133+/CD44+/ABCG2+ (Castellon *et al.*, 2012).

The discovery of CSCs has changed the way PCa tumour development is understood. (Jaworska *et al.*, 2015). Prior to their discovery, it was assumed that PCa grew via a stochastic model in which each cell in a heterogeneous cancer is able to randomly give rise to new carcinoma cells (Jaworska *et al.*, 2015). Since the discovery of CSCs, a new model has developed, the hierarchical model, in which a small population of CSCs, which do not exceed 5% of the total tumour cells, produce all the new tumour cells and are responsible for driving disease progression (Jaworska *et al.*, 2015; Leao *et al.*, 2017) (Figure 1.9).

It has also been suggested that the presence of residual CSCs may lead to recurrence and disease progression to the eventual CRPC state (Chen *et al.*, 2013). The CSC hypothesis has generated excitement as it clarifies our understanding of the heterogeneity of cancer and suggests new avenues that could be explored in the diagnosis and treatment of cancer (Guzel et al., 2014).

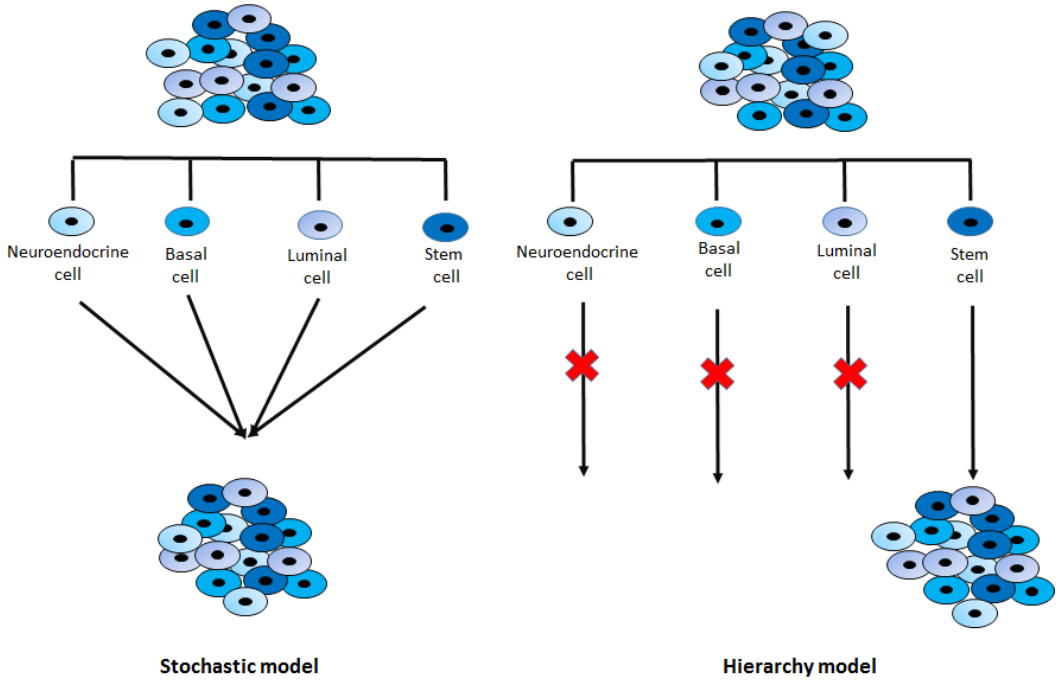

Figure 1.9 The possible models of prostate formation. In the stochastic model (left side), each cell in a heterogeneous tumour has the ability to originate new tumours when it develops the capability to self-renew. In contrast, in the hierarchical model (right side), a small number of cells called CSCs are able to originate new tumours.

1.3.3 The origins of prostate cancer and prostate cancer stem cells

The discovery of PCSCs has implications for two crucial and related questions. First, what is the cellular origin of PCa? Second, what is the cellular origin of PCSCs?

The cellular origins of PCa remain unclear, however, it has been suggested that PCa might arise from a number of different cell types (Jaworska *et al.*, 2015). The luminal cells may represent the origin of tumorigenicity in prostate because the majority of prostate epithelial cells in the tumour mass are luminal cells but, unlike the normal luminal cells, these cells are not fully differentiated cells (Jaworska *et al.*, 2015). It has been proposed that PCa could result from a more restricted cell type, such as the transit-amplifying cells (intermediate cells), which have the ability of self-renewal (van Leenders and Schalken, 2001). The basal cells have also been suggested by several studies because they have the ability to rapidly differentiate into luminal cells following oncogenic transformation (Shen and Abate-Shen, 2010). It has also been found the majority of metastatic tissues contain basal cells (Liu *et al.*, 2002), suggesting basal cells might be responsible for PCa metastasis. This model is supported by evidence from immunodeficient mice, showing that PCa can arise from basal cells in NP and this might be because basal cells are expressing oncogenes such as AR, AKT

and ERG (Goldstein *et al.*, 2010a). Finally, a study has suggested that the PSCs that are responsible for PCa formation, through their abnormal proliferation and differentiation (Takao and Tsujimura, 2008).

A related question is where do PCSCs arise from and several lines of evidence have identified three suggestions for the source of PCSCs. First, a study done by Mani *et al* suggests that CSCs are generated from cancer cells that undergo epithelial-mesenchymal transition (EMT), which represents a key development program that may contribute to invasion and metastasis (Mani *et al.*, 2008). Second, another hypothesis suggests that cancer cells may undergo a process similar to that which produces pluripotent stem cells (iPS) (Corominas-Faja *et al.*, 2013). Finally, the most common hypothesis, which is supported by several studies, suggests that CSCs originate from normal adult prostate SCs that have been exposed to oncogenic genomic alterations (Jaworska *et al.*, 2015). These mutant SCs are transformed into CSCs during the process of carcinogenesis (Jaworska *et al.*, 2015). This is supported by the fact that CSCs have most of the stem-like properties found in adult SCs, including long-lived, slow cycling, (Jaworska *et al.*, 2015), self-renewal and differentiation (Lobo *et al.*, 2007), and that they express SC markers (Collins *et al.*, 2005). This final model suggests that adult SCs are the cell type responsible for the production of CSCs and that these CSCs then give rise to all the cells of a heterogeneous tumour.

1.3.4 Stem cell biomarkers

SCs are thought to be involved in cancer initiation, progression, metastasis, recurrence and drug resistance and as a result of that, finding candidate biomarkers that can identify these cells has become an urgent necessity. It has been found that different ESC factors can be expressed in both adult SCs and CSCs such as Nanog, OCT4, SOX2 (Ben-Porath *et al.*, 2008). A previous study has found that Nanog, OCT4, Sox2 are highly expressed in tumour tissues and cancer cell lines, including colon, bladder and prostate tumours using PCR and IHC (Amini *et al.*, 2014). However, these proteins were found to have broad expression so do not appear to exclusively mark prostate SCs. Several other candidate biomarkers have been used to try and identify and distinguish PSCs and/or PCSCs from other cell types (Jaworska *et al.*, 2015), including cell surface markers: CD24, CD44 (Hyaluronic acid receptor) (Hurt *et al.*, 2008), CD49f, Trop 2 (Goldstein *et al.*, 2008) CD133 (Prominin-1 or AC133) (Collins *et al.*, 2005), CD166 (Jiao *et al.*, 2012), and α2β1 (Collins *et al.*, 2005), stem cell antigen-1 (Ssca-1) (Reiter *et al.*, 1998) and aldehyde dehydrogenase (ALDH)

(Kahlert *et al.*, 2012). These candidate biomarkers are summarised in (Figure 1.10). In addition, increased PCa proliferation rate and invasion was found in CD44+, $\alpha 2\beta 1^{high}$, CD133+ cells compared to CD133-negative cells (Collins *et al.*, 2005). Increased α2β1 expression was observed in PSCs compared to other prostate cell types in the basal compartment (Collins *et al.*, 2005) and there is increased activated leukocyte cell adhesion molecule (ALCAM/CD166) expression in castrated mice compared to intact mice (Jiao et al., 2012). ALCAM/CD166 is also expressed in a subset of adult human prostate cells as well as in PCa tissues (Ofori-Acquah and King, 2008). Jiao *et al.* found that increased CD166 expression was localised to foci in NP tissues, which overlaps with a subset of TROP2 and CD49f positive cells (Jiao et al., 2012) (Figure 1.10).

Despite the studies described above, currently no single biomarker has so been shown to be able to identify and sort PSCs and/or PCSCs, so combinations of makers are normally used (Burger *et al.*, 2005) and none of these biomarkers is used clinically in PCa diagnosis and progression. In addition, some of the most common biomarkers for CSCs, such as Sca-1, are mouse genes and don't have a direct homologue in humans (Xin *et al.*, 2005). The mouse biomarker panel is used often for basic research, but it might not necessarily translate easily into a human panel. Therefore, there is still a pressing need to find biomarkers that can identify PSCs and/or PCSCs.

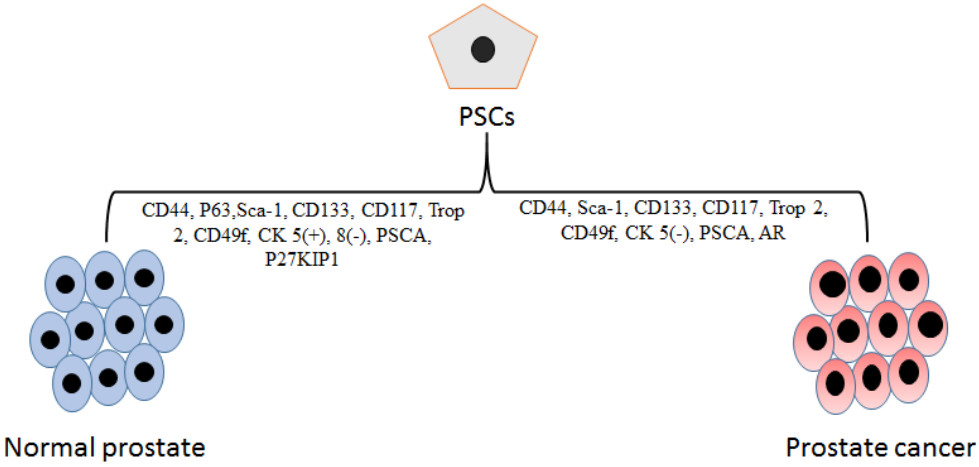

Figure 1. 10 Cellular identities of SCs in the prostate. SCs markers that are expressed in NP (left side) or PCa (right side). The majority of markers are expressed in both normal and malignant prostate tissues. However, some markers are specific to NP, including p63 and CK5 or to PCa such as AR. Adapted from (Prajapati et al., 2013).

1.3.5 Summary

SCs are cells capable of self-renewal and differentiation into specific lineages, with adult SCs capable of differentiating into a restricted range of cell types. SCs are also thought to be

the origin of CSCs, which in turn are believed to be the origin of new tumour cells and able to drive disease progression, including relapse and resistance to therapy.

Although several potential biomarkers for SCs and/ CSCs have been studied, there is still a pressing need for discovering new biomarkers that can distinguish between normal PSCs and PCSCs. If these cells are key to driving disease progression, then these potential SC biomarkers might be used to distinguish between different grades and clinical stages, such as biochemical relapse, of PCa.

1.4 Aims of the study

The primary goal of this project is to identify proteins that are differentially expressed between normal and malignant prostate tissues and/or between different grades, stages and relapsed vs non-relapsed prostate tumours. This is because differentially expressed proteins may represent new clinically useful biomarkers for PCa. Identifying proteins with differential expression is also a key step in improving our understanding of the molecular basis of prostate cancer formation and progression and may potentially help in the development of future therapies.

The aims for this thesis are;

- Select potential biomarkers to study by identifying proteins with links to SCs, the regulation of cellular differentiation and/or a suggested role in cancer initiation, progression or relapse using literature searching.
- Use IHC to determine the expression patterns of the potential biomarkers in normal and malignant prostate tissues from two independent cohorts of patients.
- Confirm the specificity of the staining with independent antibodies or alternative staining approaches.
- Test if the expression of these candidate biomarkers correlates with clinical features of PCa, including primary Gleason grade and clinical stage in both cohorts.
- Decide if any of the proteins could be potential clinical biomarkers or warrant future study to understand their role in PCa

CHAPTER TW0
MATERIALS AND METHODS

2. Materials and Methods

2.1 Materials

All general laboratory chemical reagents were purchased from Sigma-Aldrich, Fisher Scientific UK Ltd, DAKO and Advanced Cell Diagnostic (ACD) unless otherwise specified. Room temperature was the standard temperature that was used to store all buffers and solutions unless otherwise stated.

2.1.1 Patient samples

In this retrospective study, two different sources of prostate samples were used, including a Bath and a tissue microarray (TMA) cohorts. This work was covered by the National Health Service (NHS) ethical and research approval (REC reference: 13/WS/0153; IRAS project ID: 112241). The composition of the two cohorts of tissue is summarised below and the clinical characteristics are described in more detail in Chapter 3 for the Bath cohort and Chapter 4 for the TMA cohort.

2.1.1.1 Bath cohort samples

A total of 34 paraffin-embedded blocks of PCa, including those from patients who had, and had not, undergone recurrence, and 5 NP tissue samples, were obtained from the histopathological laboratories of the Royal United Hospital (RUH), Bath/ UK. The samples were collected between 1997 and 2018. This study also used normal testis, placenta, liver and kidney tissue samples obtained from the RUH as positive controls. This cohort is described in details in Chapter 3.

2.1.1.2 Tissue microarray cohort samples

TMA slides were obtained from US Biomax (PR1921). Each tissue array slide had 96 cases, 80 of them were a prostate carcinoma, whereas, the rest were normal or normal tissues that were adjacent to the PCa, termed adjacent normal (8 cases for each). Each case was represented with two core tissue biopsies to form a total of 192 cores. This cohort is described in details in Chapter 4.

2.1.2 Primary antibodies

Details of the primary antibodies used in this study, including the supplier, catalogue number, species and dilution are shown in Table 2.1.

Table 2. 1 The antibodies used in this study.

Primary antibody	Supplier	Type of antibody	Catalogue no.	Antigen retrieval	Applied dilution
ABCG2	Abcam	Mouse monoclonal	Ab3380	Citrate buffer (pH6)	1:100
ALDH1A1	Abcam	Rabbit monoclonal	Ab52492	Citrate buffer (pH6)	1:100
NDRG1	Abcam	Rabbit monoclonal	Ab124689	Citrate buffer (pH6)	1:250
RS1	Sigma-Aldrich	Rabbit polyclonal	SAB1100315	Citrate buffer (pH6)	1:500
Sall4	Novus bio	Mouse monoclonal	H00057167-M03	Tris/ EDTA (pH9)	1:100
Sall4	Abcam	Rabbit polyclonal	Ab29112	Tris/ EDTA (pH9)	1:100
Sall4	Abcam	Rabbit monoclonal	Ab181087	Tris/ EDTA (pH9)	1:50
Sox7	Abcam	Rabbit polyclonal	Ab80331	Citrate buffer (pH6)	1:200
Sox7	Sigma	Rabbit polyclonal	A40200	Citrate buffer (pH6)	1:200
Zscan4	Novus biological	Mouse polyclonal	H00201516-B01P	Citrate buffer (pH6)	1:200
Zscan4	Abcam	Rabbit polyclonal	Ab106646	Citrate buffer (pH6)	1:200
β-catenin	Cell Signaling Technology	Rabbit polyclonal	95625	Citrate buffer (pH6)	1:100

2.1.3 mRNA Probes

Details of the mRNA probes used in this study, including supplier and catalogue number are shown in Table 2.2.

Table 2. 2 The mRNA probe used in this study.

Probes	Catalogue no.	Supplier
Hs- Sall4	5050701	ACD
Hs-Zscan4	421091	ACD
Positive control (Hs-PPIB)	313901	ACD
Negative control (Dap B)	310043	ACD

2.1.4 Additional materials

Other materials that were used in this study are as described in Table 2.3

Table 2.3 Additional materials used in this study.

Materials	Supplier	Catalogue no.
3-Aminopropyl triethoxysilane	Sigma Aldrich	A3648-100 mL
Acetone	Sigma- ALDRICH	24201-2.5 L.R
Ammonia solution	BDH laboratory supplies	452394U
Antibody diluent	DAKO	S0809
Bovine serum albumin (BSA)	Sigma	A4503-50g
Compound microscope with digital image accessories	Nikon	E800
Coverslips (22x50)	Scientific laboratory supplier	NO 1
Dako Envision™ +system-HRP (DAB)	Dako	K4010
Dako liquid DAB+ substrate chromogen system	DAKO	K3468
DPX mountain for histology	Sigma	06522-100mL
Eosin	Raymond Alamb	S1153
Ethanol Absolute	VWR chemicals	20821 330
Glass slides	VWR	631 - 1551
Haematoxylin	Vector	H-3401
HCL	Sigma Aldrich	25,812-8
Histoclear	National diagnostic	H5-200
Hybez II oven	ACD	240200ACD-2
Immuneedge pen	Vector Laboratories	H-4000
MB22 Premier Microtome blade	Thermo Scientific	12647896
Microtome	Leica RU	2155
Normal Goat Serum (NGS)	Sigma	G9023-10ml
Phosphate buffered saline (PBS) tablets	OXOID	BR00140
Prostate cancer tissue microarray slide	US Biomax	PR1921
RNAscope® control slides- human HeLa cell pellet	ACD	310045
RNAscope® H_2o_2 & protease pulse reagents	ACD	322330
RNAscope® target retrieval reagents	ACD	322000
RNAscope® wash buffer reagents	ACD	310091
RNAscope® 2.5. HD detection reagent –Brown	ACD	322310
Sodium hydroxide	Sigma Aldrich	S8045-500g
Tri-sodium citrate	Sigma- ALDRICH	S1804-500ML
Tris (hydroxymethyle) methylamine	Fisher scientific	T/p630/90

Triton X100	Sigma	T8787-100ML
Tween 20	Sigma	P9416-100ML

2.1.5 Preparation of reagents

In this study, numerous reagents were used as a part of both IHC and RNAscope® methods. The methods used to prepare these reagents are described in Table 2.4.

Table 2. 4 The reagents used in this study with the methods of preparation.

Reagents	Preparation method
1 x and 10x PBS solution pH 7.4	To prepare 1x PBS, 1x PBS Dulbecco's tablet was dissolved in 100 mL of distilled water (ddH_20), whereas, 10 tablets of Dulbecco's PBS (Thermo Scientific) were dissolved in 100 mL of ddH_20 to prepare10x PBS. These solutions were then sterilised by the autoclave.
2% (3 Aminopropyl) triethoxysilane (APTS)	10 ml of silane was dissolved in 500 mL acetone. This solution is unstable and was stored for 8 hours only.
50% Haematoxylin staining solution	100 mL Gill's Haematoxylin was added to 100 mL ddH_20 in a dish. The solution was then filtered by a filter paper.
70% Ethanol	30 mL ddH_20 was added to 70 ml of absolute ethanol.
90% Ethanol	10 mL ddH_20 was added to 90 ml of absolute ethanol.
95% Ethanol	5 mL ddH_20 was added to 95 ml of absolute ethanol.
Ammonia water	1.43 mL of ammonium hydroxide was added to 250 mL ddH_20, then mixed well, and then stored at room temperature.
Blocking buffer	10% NGS and 0.5% BSA were added to PBS and then mixed well, using a roller mixer to dissolve completely. Blocking buffer was then stored in the fridge at 4°C before using.
Bluing solution	1% NH_3 was added to 70% ethanol.
Citrate buffer (pH 6)	To prepare a citrate pH6 buffer, 2.941 gm of tri-sodium citrate was dissolved in 1L ddH_20. The pH of the solution was then adjusted to pH6 by adding few drops of HCl. 500µL of Tween-20 then was added to the solution and mixed well to dissolve completely and then stored at room temperature.
Differentiate solution	I% HCl was added to 70% ethanol.
Permeabilisation solution	0.5% of Triton was added to 1x PBS and then mixed well.
Phosphate buffer saline with 0.05% tween 20 (PBST)	10 PBS Dulbecco's tablets were dissolved in 1L ddH_2O and mixed well. 500 µl of Tween 20 then was added to the solution. Finally, this solution was dissolved completely by using a magnetic stirrer.

RNAscope® 1X Target Retrieval Reagents	20 mL 10X target retrieval reagent was added to 180 mL ddH_20, then mixed well. This solution was then stored at room temperature before using.
RNAscope® 1X Wash Buffer	To prepare 3L of 1X wash buffer, a bottle (60 mL) of RNAscope® washing buffer (50X) was warmed up to 40 °C for 10-20 minutes, then added into 2.94 L ddH_20 and mixed well. This solution was then stored at room temperature.
Tris/EDTA buffer (pH9)	To prepare a Tris / EDTA pH6 buffer, 1.21 g of Tris hydroxymethyl methylamine and 0.37223 g of ethylenediaminetetraacetic acid disodium salt dihydrate were added into a litre of ddH_2O and stirred to dissolve, then the pH of the solution was adjusted to pH9 by adding few drops of sodium hydroxide. 500µL of Tween-20 was also added to the solution and mixed well to dissolve completely. This solution was stored at room temperature before using.

2.2 Methods

There are different methods used in this study, including silane coating of slides, H&E, IHC, and RNAscopes® are described in detail below.

2.2.1 Coating Slides with (3-aminopropyl) triethoxysilane

APTS coating is a method used to enhance the adherence of tissue sections on the microscope slides to avoid lifting of the tissue from slide especially during the antigen retrieval step in both IHC and RNAscope®. The APTS coating procedure had three different steps: First, the slides were cleaned by immersion in absolute acetone for 2 minutes, then left to dry in a fume hood. Second, the dried slides were then dipped for two minutes into a 2% solution of APTS that has been prepared previously (see Table 4.2). Finally, the slides were washed twice with absolute acetone for 2 minutes each, then left to dry completely.

2.2.2 Sectioning

Formalin-fixed paraffin-embedded (FFPE) prostate tissue blocks were chilled in a fridge at 4°C before using and then 5µm thick sections were cut by using a microtome blade mounted in a microtome from Leica RU Company. The cut sections were then put into a water bath at 37°C to allow them to flatten and then picked up onto microscope slides. Slides were then dried and baked overnight at 37°C on a hot plate. Next day, the baked slides were then stored at room temperature to be used later.

2.2.3 Haematoxylin and Eosin staining protocol

H&E staining is a common method used to highlight the morphological architecture of tissues under the light microscope and can be used to detect the abnormal changes in the tissue architecture that may be observed in many diseases, including cancer. H&E was carried out as follows:

Two pre-treatment steps were carried out prior using H&E staining. First, a dewaxing step was performed by soaking the slides into two changes of Histoclear for 2 minutes each to remove the paraffin from tissues. Second, rehydration of tissue sections was then carried out using two washes in each of a graded series of ethanol solutions (100%, 95%, 90%, 70% and 50% respectively) for a minute each time and then rinsed with ddH_20 for 2 minutes.

After the pre-treatment steps described above, the tissue sections were then ready to stain, using haematoxylin which is used to stain the nucleus of cells. In this step, drops of haematoxylin were added onto tissue sections for 2 minutes and then rinsed under tap water for three minutes. To differentiate the haematoxylin stain, the slides were then soaked three times in 70% ethanol with 1% HCl. The slides were also immersed for a minute in an alkaline solution that was prepared by adding 1% ammonium hydroxide to 70% ethanol to restore the blueing stain of haematoxylin. Drops of 0.5% eosin in ddH_20 then were added to slides for three minutes to stain the rest of tissue architectures. At this point, the staining steps were finished.

After that, the slides were washed with two changes of different ethanol concentration 95% and 100% for 15 seconds and 1 minute, respectively. Slides were then washed twice with Histoclear for 2 minutes each. Three drops of DPX mounting media were applied onto the slides and then these slides were covered with coverslips and left to dry. Next day, the slides were ready to examine under a light microscope (Nikon Eclipse E800) equipped with a Nikon digital camera (DS-U1 CCD).

2.2.4 Immunohistochemistry assay

IHC is a method used to identify specific tissue components, including proteins and is routinely used in cancer research. This assay can be completed within two days, and the procedure was carried out as follow:

Prior to IHC, pre-treatment steps, including deparaffinization and rehydration, were necessary to remove the paraffin from tissues and to rehydrate tissue samples respectively.

A dewax step was performed by soaking the slides into two changes of Histoclear for 7 minutes each. Rehydration of tissue sections was then carried out using two washes with a graded series of ethanol solutions (100%, 95% & 70% respectively) for a minute each time. These slides were then immersed in ddH_20 for 2 minutes, followed by two times PBS washing for 5 minutes each. Tissues were then permeablised by adding 0.5% Triton X-100 in PBS for 30 minutes. After that, the sections were washed three times with PBS for 5 minutes each.

To carry out the antigen retrieval, tissue sections were pre-treated in a water bath at 90 ^{0}C for 30 minutes with heat-induced epitope retrieval buffer, citrate or Tris/EDTA, at pH 6 or 9 respectively. These sections were left to cool inside the retrieval buffer jar at room temperature for 20 minutes and then washed twice with PBS for 5 minutes each. The slides were then allowed to dry. The tissue sections were circled using an ImmunEdge hydrophobic barrier pen to be sure that reagents covered the whole tissue sections on the slides. To remove the endogenous peroxidase activity, drops of peroxidase block were added onto the tissue sections, then the slides were placed in a humid chamber and incubated at room temperature for 10 minutes, followed by rinsing gently three times with PBS buffer for 5 minutes each. Drops of blocking buffer that had been prepared previously (Table 2.4) were then added onto tissue sections and incubated in the humid chamber at room temperature for 30 minutes, followed by two times PBS washing for a minute each. At this point, the pre-treatment steps were finished.

After finishing the pre-treatment steps described above, the tissue sections were ready to stain with a DAKO EnVision IHC staining kit. IHC had three main steps; primary antibodies were diluted to the appropriate dilution (see Table 2.1) using Dako antibody diluent, and 100-150 µM of diluted primary antibodies were applied to each section and incubated in a humid chamber at 4^{0}C overnight. The next day, slides were washed three times with PBS buffer for 10 minutes each. Drops of goat anti-mouse or goat anti-rabbit labelled polymer – HRP conjugated secondary antibodies were added onto the slides and then incubated at room temperature with gentle rocking for 30 minutes. Slides then were washed three times with PBS buffer for 5 minutes each. To prepare the chromogenic reagent solution that was used to visualize the antigen-antibody reaction, a drop of DAB chromogen was added into 1mL of the substrate buffer and then mixed well. After preparation, drops of the prepared solution were added onto the tissue sections and then incubated in the humid chamber for 5 minutes at room temperature. To terminate the chromogenic reaction, slides then were washed three

times with ddH_2O for 5 minutes each. Drops of the Vector haematoxylin solution, as a counterstain, were applied and then incubated at room temperature for a minute to stain the nucleus of cells. Slides then were rinsed thoroughly under running tap water for 3 minutes.

The rest of the IHC steps, including differentiation and bluing of the haematoxylin, rehydration, Histoclear, mounting and slides examination was required, were carried out as described above. 5 random images were taken using Nikon Digital camera (DS-U1 CCD) attached to a Nikon Eclipse E800 microscope.

2.2.5 RNAscope®

RNAscope® is a new method used to identify specific tissue components, including mRNA. This study used a RNAscope® 2.5 HD assay-brown kit which uses ACD patented signal amplification and background suppression technology. This method uses a novel double Z probe that binds to the target RNA and then bound to a cascade of a preamplifier, amplifier, and label probe (Wang *et al.*, 2012). The version of RNAScope® that was used in a uniplex assay that detects individual mRNA molecules as brown dots in the cells and this assay can be completed within two days, and the procedure was carried out as follows:

The RNAscope® assay was carried out following manufacturer instructions and as described below: During the first day, slides were baked in a dry oven for an hour at 60°C, and during this time all reagents were prepared. After baking, slides were soaked two times in fresh Histoclear for 5 minutes each. Tissues were then washed with two absolute ethanol changes for a minute each. Slides were allowed to dry at room temperature for 5 minutes. To block any endogenous peroxidase in the tissue, 5-8 drops of H_20_2, a first pre-treatment, was applied on the tissue sections for 10 minutes at room temperature. Slides were then washed by moving the rack up and down 3-5 times and repeated washing with fresh ddH_20. Target retrieval, a second pre-treatment, was then carried out. The slides were placed in a heat-resistant jar with the 1x target retrieval solution and then heated at 98-100°C on a hot plate for 15 minutes. The slides rack was immediately transferred from a hot plate to a jar containing ddH_20 and then washed several times by moving the rack up and down, followed by a fresh 100% ethanol washing and then allowed to air dry. Finally, tissue sections were circled using an ImmunEdge hydrophobic barrier pen and allowed to dry overnight.

Next day, a Hybez II oven from ACD was set to 40°C to be used to warm the probes for 10 minutes to ensure any salts in probe diluent that may have precipitated were dissolved then allowed to cool at a room temperature before use and RNAscope® amplifier reagents were

also moved from the fridge and then kept at room temperature before use. After the preparation steps mentioned above, drops of protease pulse, a third pre-treatment, was added onto the tissue sections and then incubated in the humid sealed tray containing HybEZ™ Slide rack at 40°C for 30 minutes to remove RNA-binding proteins. This makes it easier for the probes to bind to the mRNA transcripts and also permeablises the tissue/cells to enable good penetration and reduce trapping of any of the reagents used in the assay. After that the slide rack was moved from the oven, then slides were washed many times by moving the rack up and down in ddH_20.

Drops of mRNA probes were added onto tissue sections and then incubated in a humid tray inside the oven at 40 °C for 2 hours. Slides were washed twice with 1x wash buffer for two minutes each. Six amplifiers (1-6 AMP) steps then were applied to the tissue sections, and then incubated in a sealed tray containing HybEZ™ Slide rack at 40 °C for 30, 15, 30, 15, 30, 15 minutes respectively. Each amplifier step was followed by two times washing with buffer solution for 3 minutes each.

To detect the signal, DAB was prepared by adding an equal amount of brown A & B and mixed well. 120µl of the DAB solution was dropped onto the tissue sections and incubated in a sealed tray containing HybEZ™ on a slide rack for 10 minutes at RT. Slides were washed by moving the rack up and down inside a ddH_2O jar. To stain the nucleus of cells, 50 % haematoxylin solution were applied onto tissue sections and incubated at room temperature for 2 minutes. Slides then were rinsed thoroughly with running tap water for 2 minutes, followed by 0.02% ammonia water washing for a few seconds. After that, slides were washed twice with different alcohol concentrations, 70% and 100%, for a minute each time. These slides were then washed twice with Histoclear for 5 minutes per change. Three drops of DPX mounting media were applied onto the slides and then these slides were covered with coverslips and left to dry. Finally, the slides were examined under a light microscope and 5 random images were taken using Nikon Digital camera slight (DS-U1 CCD) that attaches with a Nikon Eclipse E800 microscope.

2.3 IHC Quantification

Prior to quantification, 5 random fields were taken at 20x magnification for each potential biomarker. The images were then scored using one or more of a number of different scoring systems to quantify the expression of the candidate proteins in the epithelial, and in some

cases, the stromal cells of the prostate tissues. The most appropriate scoring system for IHC was selected for each biomarker based on the previous studies.

2.3.1 H-Score method

The H- Score method is one of the most common scoring systems that are normally used to score the protein signal in IHC. The score given depends on the proportion of positive cells and staining intensity. To carry out the scoring, nuclei of epithelial cells were counted based on four DAB staining intensity categories (0: No nuclear staining 1: Weak nuclear staining, 2: Moderate nuclear staining and 3: Strong nuclear staining). After scoring, the H-score was calculated as the following formula:

H score= 3 x % of strongly nuclear staining + 2 x % of moderately nuclear staining + % of weakly nuclear staining.

The range of H-score can vary between 0 and 300 (Aye Thike, 2001). This system was used to score the nuclear expression of β- catenin, Sall4 and Zscan4 only.

2.3.2 Other IHC scoring methods

Six other types of semi-quantitative scoring were used to score the expression patterns of candidate proteins in the tissue sections. The scoring system (proportion and intensity 1) was used unless otherwise specified. The alternative scoring system was used when the previous studies were used in the specific scoring system. The full details were shown in Table 2.5.

Table 2. 5 The scoring systems used for each antibody.

Name	proteins	Scoring system	References
Proportion and intensity 1	Nuclear and cytoplasmic NDRG1, ABCG2, RS1 and the cytoplasmic β-catenin and Zscan4	• The Percentage of positive cells was scored as: (0: 0; 1: 1-25%; 2: 26-50%; 3:51-75%; and 4: 76-100%). • The intensity was graded as (0: negative, 1: weak, 2: moderate; and 3: strong). • The final score represents the sum of the proportion and intensity scores, which ranged from 0 to 7.	(Dalley *et al.*, 2014)
Proportion and intensity 2	Sox7	• The proportion of stained cells (nuclear and cytoplasmic) was scored as follows: (0: negative, 1: 1-10%, 2: 11-50 % and 3: 51-100 %.).	Adapted from (Zhong *et al.*, 2012).

		• The intensity of nuclear and cytoplasmic staining was scored as follows: (0: negative, 1: weak, 2: moderate and 3: strong). • The final score represents the sum of proportion and intensity scores of cytoplasmic and nuclear staining, which range from 0-6.	
Localisation 1	NDRG1 localization	The localization of NDRG1 was scored as Pattern 1: predominantly in the cell membrane. Pattern2: predominate in nucleocytoplasmic. Pattern 3: not detectable expression.	Adapted from (Caruso *et al.*, 2004; Hosoya *et al.*, 2013)
Proportion and intensity 3	Nuclear and cytoplasmic ALDH1A1	• The proportion of stained cells (nuclear and cytoplasmic) was scored as follows: (0: negative, 1: 1-33%, 2: 34-65 % and 3: 66-100 %.). • The intensity of nuclear and cytoplasmic staining was scored as follows: (0: negative, 1: weak, 2: moderate and 3: strong). • The final score represents the sum of proportion and intensity scores of cytoplasmic and nuclear staining, which range from 0-6.	Adapted from (Matsika *et al.*, 2015).
Proportion 1	Stromal ALDH1A1 proportion staining	• The proportion of stromal stained cells was scored as follows: (0: negative, 1: 1-33%, 2: 34-65 % and 3: 66-100 %.).	Adapted from (Matsika *et al.*, 2015).
Intensity 1	ALDH1A1 stromal intensity and cytoplasmic RS1 (Bath cohort only)	The intensity of nuclear and cytoplasmic staining was scored as follows: (0: negative, 1: weak, 2: moderate and 3: strong).	Adapted from (Matsika *et al.*, 2015).

2.4 RNAscope® quantification

Two different scoring systems were used to quantify the Sall4 and Zscan4 mRNA levels in the prostate tissue samples. For both systems, 5 random fields were taken at 20x magnification and then the number of brown dots in the prostate cells was scored. Scoring of RNAScope used manufacturer recommended approaches to score mRNA sall4, using five different criteria depending on the number of brown dots on prostate epithelial cells. The

modified scoring system was used to score Zscan4 mRNA because the number of positive cells and brown dots were few. The different scoring systems and which candidates they were used for are described in detail below in Table 2.6.

Table 2. 6 The scoring systems used for each mRNA probe.

mRNA probe	Scoring system	References
mRNA Sall4	The positive cells were scored as : 0: no staining 1: 1-3 dots/cell 2: 4-9: dots/ cell 3: 10-15 dots/cell and <10% dots are in clusters 4: >15 dots/cell and >10% dots are in clusters	Adapted from ACD https://acdbio.com/technical-support/solutions 20/10/2017
mRNA Zscan4	The positive cells were scored as : 0: no staining 1: 1-10% positive cells 2: 11-30% positive cells 3: 31-100% positive cells	Modified according to advice by email from Dr Bradley Spencer-Dene, Technical Support/ FAS Europe, ACD. 21/7/2017.

2.5 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com, including mean, standard error and standard deviation values as well as the other statistical analysis such as a frequency distribution test and histogram. Statistical analysis was carried out either using unpaired T-test, Chi-Squared and /or one-way ANOVA with Tukey's multiple comparisons tests. Results were considered significant if the P.value was ≤ 0.05.

CHAPTER THREE

IDENTIFICATION AND ASSESSMENT OF CANDIDATE PROTEINS IN PROSTATE TISSUES FROM THE BATH COHORT

3. Identification and assessment of candidate proteins in prostate tissues from the Bath cohort

3.1 introduction

The primary goal of identifying new biomarkers for PCa is to improve diagnostic and prognostic accuracy. Identification of potential new biomarkers that can help to detect PCa in early stage and/or distinguish between an aggressive tumour requiring radical intervention and those that have a good prognosis is the long term goal of this study. The first step in this process is to identify the proteins whose expression would be examined. The criteria used to select proteins was a link to SCs and the regulation of cellular differentiation and/or a suggested role in cancer initiation, progression or relapse. The second step was to determine the association between the staining patterns of the potential biomarkers and PCa clinical parameters, including primary Gleason grade, clinical stage and relapse, using tissues from the Bath cohort of patients. This introduction will review the different approaches that can be used for biomarkers identification and set out the aims for this chapter.

3.1.1 Approaches to identify potential biomarkers for further analysis

Identification of biomarkers was the first step in this study and there are a number of approaches that can be used. Each approach has advantages and disadvantages which are reviewed below.

3.3.1.1 Transcriptomics

Transcriptomic is an approach that investigates the transcriptome changes in tumour samples to identify new biomarkers in a large cohort, using cDNA microarray or high throughput RNA sequencing (Long *et al.*, 2014; Sowalsky *et al.*, 2015; Trost *et al.*, 2015; Lowe *et al.*, 2017).

Previous studies used transcriptomic methods to identify potential new biomarkers for cancer, including PCa. For example, Long *et al* reported 24 biomarkers identified to predict PCa relapse, using a global transcriptomic analysis of formalin-fixed PCa samples (Long *et al.*, 2014). Another study reported that a series of un-spliced mRNA was found to predict metastatic CRPC, using whole transcriptomic sequencing (RNA seq) (Sowalsky *et al.*,

2015). This approach had some success in identifying genes that were expressed differently in different grades of cancer, including breast and prostate tumours (Ben-Porath *et al.*, 2008; Long *et al.*, 2014). Despite the advantages of transcriptomic technique in identifying new biomarkers, this technique, however, is a complex method and also needs high throughput approaches and costly materials to assess patient samples.

3.3.1.2 Proteomics

Proteomics represents an approach used to study the structure and function of proteins in the biological samples (Chandramouli and Qian, 2009). This approach is used to identify potential biomarkers by measuring the protein levels in tissue (Adeola *et al.*, 2017; Shields and Wu, 2018), serum (Larkin *et al.*, 2016; Adeola *et al.*, 2017; Chandramouli and Qian, 2009) and urine (Beasley-Green, 2016) samples, using different techniques, including IHC and Enzyme-linked immunosorbent (ELISA).

There are several publications that have used this method for biomarker identification. For example, a previous study used proteomics successfully to identify sex biomarkers which could be used for PCa diagnosis from high-quality serum samples (Larkin *et al.*, 2016). Another proteomic study identified thousands of proteins from BPH and PCa samples, including macrophage inhibitory cytokine-1, which was found to be increased in PCa compared to PBH cells (Hood *et al*., 2005). In contrast, Kim *et al* failed to identify a single biomarker that could be used to distinguish between PCa subgroups from prostate fluid and urine samples, using proteomics analysis (Kim *et al.*, 2016).

It can be seen from the previous studies that this approach has several advantages; including providing a wealth of the potential biomarkers that could be used for further follow-up studies. However, like transcriptomics, it is a complex method that needs expensive equipment and costly materials to assess patient samples.

3.3.1.2 Literature searching

Literature searching represents perhaps the simplest approach for identifying new potential biomarkers for further analysis. In this approach, a series of keyword searches are carried out and returned the literature reviewed to identify proteins warranting analysis.

This approach has many advantages and disadvantages. One advantage is that there is no requirement for knowledge of text mining or specific methods or equipment (Mahood *et al.*,

2014). In addition, this approach can be exhaustive and time-consuming, but it does not require any laboratory work compared to the previous methods that require extensive laboratory work and need time to accomplish (Grewal *et al.*, 2016). However, the investigator needs to use their knowledge or experience to select and then assess the literature used to make the decision and it relies on existing information, no new experimental results are generated to inform the decision.

This study used the literature searching approach as a simple and affordable method for identifying new potential biomarkers that link to SCs and the regulation of cellular differentiation and/or a suggested role in cancer initiation, progression or relapse. IHC protocols for the markers can then be established and the expression of these potential biomarkers assessed in normal and malignant prostate tissues. In this chapter, this will be carried out using samples collected at the RUH in Bath city/UK.

3.1.2 Aims

- Identify proteins that have links to SCs, the regulation of cellular differentiation and/or a suggested role in cancer initiation, progression or relapse using literature searching.
- Establish IHC staining protocols for the proteins of interest.
- Determine the expression patterns of the potential biomarkers in normal and malignant prostate tissues from the Bath cohort patients.
- Test if the expression of these candidate biomarkers correlates with clinical features of PCa, including primary Gleason grade, clinical stage and relapse.

3.2 Results

3.2.1 Identification of potential biomarkers using literature searching

Identification of potential biomarkers that warranted further analysis was the first step in this study. A literature searching approach was carried out using PubMed (https://www.ncbi.nlm.nih.gov/pubmed. Data accessed: June 2015) and the criteria for selection included a link between the protein of interest and SCs and the regulation of cellular differentiation and/or a suggested role in cancer initiation, progression or relapse. The research began with searches using terms including “prostate cancer recurrence or relapse”,

"cancer recurrence or relapse", "prostate cancer stem cells", "stem cell biomarker", "stem cell biomarkers or cancer stem cell biomarkers". The searches returned publications containing hundreds of potential biomarkers and eight were selected for evaluation in this thesis. Five potential biomarkers are examined in this Chapter and the rationale for selection, against the criteria described above, is described below and summarised in (Table 3.1). The three other potential biomarkers are described in Chapters 5, 6 and 7.

A hypothesis describing the predicted changes in expression was proposed for each potential biomarker. This hypothesis was based on the expression changes that were supported by the literature. In some cases, conflicting evidence meant a number of hypotheses could have been proposed, with these, the hypothesis used were selected based on the expression changes that seemed best supported by the existing literature, for example, changes supported by more than one study or by studies with larger sample sizes. These hypotheses were then tested by examining the expression of these proteins in the Bath cohort (This Chapter) and a larger TMA cohort (Chapter 4).

3.2.1.1 β-Catenin

β-catenin is a multifunctional protein that plays an important role in cadherin-mediated adhesion and the Wnt signalling pathway (Willert and Nusse, 1998; Bismar *et al.*, 2004). Wnt/ β-catenin pathway dysregulation is found to be associated with many human diseases, including a range of cancers (Kim *et al.*, 2013). Of particular importance for this study, altered expression levels and localisation (nuclear vs. cytoplasmic) of β-catenin has been suggested to play a role in PCa progression and relapse (described below and summarised in Table 3.1).

There are several publications that have studied β-catenin level and localisation in PCa compared to NP and/or BPH, however, in many cases, these studies had different findings. In terms of nuclear β-catenin staining, a recent study found that nuclear β-catenin localisation was increased significantly in PCa compared to BPH (Ipekci *et al.*, 2015). In contrast, other studies showed decreased nuclear staining or localisation of β-catenin significantly in PCa compared to BPH (Horvath *et al.*, 2005; Whitaker *et al.*, 2008). Importantly, the Whitaker *et al* study had a larger sample size (225) than the recent Ipekci *et al* paper. Another study with a small number of PCa samples (17) showed no significant staining for β-catenin in PCa tissue samples (Bismar *et al.*, 2004).

In addition to nuclear β-catenin staining, membranous and cytoplasmic β-catenin staining has been examined in cancerous and noncancerous prostate tissues. For example, one study found that membranous β-catenin staining was reduced significantly in localized PCa compared to BPH, whereas, cytoplasmic β-catenin staining did not show a significant difference between benign and malignant prostate groups (Horvath *et al.*, 2005). Other studies found membranous β-catenin expression was reduced in PCa compared to BPH (Bismar *et al.*, 2004; Jaggi *et al.*, 2005).

The published evidence is also complicated and often at least partially contradictory for PCa Gleason grades. Nuclear β-catenin expression was found to be reduced significantly in PCa tissues with a high Gleason grade compared to those with a low grade (Whitaker *et al.*, 2008). In addition, other studies found membranous staining of β-catenin in PCa significantly reduced with increasing Gleason grades (Kallakury *et al.*, 2001a; Jaggi *et al.*, 2005; Aaltomaa *et al.*, 2005). In contrast, increased nuclear β-catenin staining was significantly associated with increasing Gleason grade (Jaggi *et al.*, 2005). This study, however, had a small number of PCa cases (17 cases). Other data showed no significant association between β-catenin staining patterns or nuclear localisation with Gleason grades (Horvath *et al.*, 2005; Ipekci *et al.*, 2015). Therefore, these studies came to different conclusions about the changes that occur in β-catenin expression and localisation in different Gleason grades.

There are a large number of published studies that describe the association between β-catenin expression and PCa stage. For example, nuclear β-catenin staining was found to be significantly increased in advanced PCa (T3-4) compared to localised disease (T3-4) (Aaltomaa *et al.*, 2005; Ipekci *et al.*, 2015). In contrast, a small sample size study reported no association between nuclear or membranous β-catenin expression and PCa stage (Jaggi *et al.*, 2005). Finally, Horvath, *et al.* found that patients who had an advanced PCa had a significant reduction in nuclear β-catenin staining compared to those with a localised PCa (Horvath *et al.*, 2005). This study had the largest cohort suggesting that the results are more likely to be reliable than some of the studies based on smaller cohorts.

Finally, in terms of a link to biochemical relapse, a reduction of nuclear β-catenin localisation has been suggested to be significantly associated with a higher risk of biochemical relapse (Horvath *et al.*, 2005). In addition, Whitaker, *et al*. found reduction of nuclear β-catenin localisation in hormone recurrent PCa compared to non-recurrent, but the data was not significant (Whitaker *et al.*, 2008). In contrast, there was no association between

membranous β-catenin expression and PCa biochemical relapse (Kallakury *et al.*, 2001; Horvath *et al.*, 2005).

Given the conflicting evidence, described above, it was decided that it would be worthwhile to re-examine the expression of β-catenin in PCa. Specifically, to examine nuclear β-catenin expression in normal and malignant prostate tissues, including recurrent and non-recurrent. Nuclear staining was selected as it appeared to have the strongest evidence to support a link between it and PCa formation and progression. Cytoplasmic staining was also analysed to provide a comparison to the results obtained for nuclear staining. It was decided not to study membranous staining as there were fewer links between changes in membranous expression and disease. This localisation could be examined in future.

The conflicting evidence meant a number of hypotheses could have been proposed, the ones used in this study were mainly based on the studies with the larger sample sizes as it was felt they were more likely to be reliable. The hypothesis used proposed that that nuclear, but not cytoplasmic, β-catenin staining will be decreased in PCa compared to NP and will be negatively associated with histopathological parameters of PCa, including primary Gleason grade, stage and relapse.

3.2.1.2 NDRG1

NDRG1 is an intracellular protein that is thought to be a tumour suppressor that plays a role in inhibiting cell proliferation and invasion (Li *et al.*, 2015b). NDRG1 expression and/or localisation has been studied in several kinds of malignancy, including PCa. Of particular importance for this study, altered expression levels and localisation of NDRG1 has been suggested to play a role in PCa progression and relapse (described below and summarised in Table 3.1).

NDRG1 expression and/or localisation have been studied in both normal and malignant tissues. Cytoplasmic NDRG1 expression was increased significantly in a range of cancer types, including colorectal (Koshiji *et al.*, 2007), hepatocellular (Angst *et al.*, 2006; Sibold *et al.*, 2007), thyroid (Gerhard *et al.*, 2010) and prostate tumours (Symes *et al.*, 2013) compared to normal or benign tissues. In contrast, a recent study showed reduced cytoplasmic NDRG1 significantly in PCa tissues and PCa cell lines compared to BPH and NP cell lines, respectively (Li *et al.*, 2015b). In terms of NDRG1 membranous expression, a previous study showed decreased membranous NDRG1 expression in PCa compared to NP tissues (Song *et al.*, 2010). NDRG1 expression has been reported in the nucleus of NP

tissues (Li *et al.*, 2015) and in a small number of PCa cases (11 of 148 cases) (Song *et al.*, 2010). However, to our knowledge, if there is an association between levels of nuclear NDRG1 expression in normal vs. malignant tissues, it has not been investigated before. Therefore, there is evidence to suggest that altered cytoplasmic/membranous, but not nuclear, NDRG1 expression is linked to PCa.

The published evidence on NDRG1 expression in different Gleason grades is also complicated. For example, there was no significant association reported between NDRG1 expression and Gleason grade (Caruso *et al.*, 2004; Symes *et al.*, 2013). In contrast, other publications showed that there was an association between NDRG1 staining and cancer grade, including PCa. For example, a previous PCa study in 2010 found increased cytoplasmic NDRG1 expression significantly associated with increasing Gleason grades (Song *et al.*, 2010), whereas, Bandyopadhyay *et al*. reported decreased cytoplasmic NDRG1 expression significantly with increasing Gleason grades (Bandyopadhyay *et al.*, 2003). Membranous NDRG1 staining was also detected in PCa and was negatively associated with increasing Gleason grade (Song *et al.*, 2010). There was no previous study that studied nuclear NDRG1 expression in normal vs. malignant prostate tissues. A study on renal cell carcinoma tissues showed that nuclear NDRG1 staining was negatively associated with increasing grades (Hosoya *et al.*, 2013). Therefore, there is conflicting evidence to suggest that changes in nuclear, membranous and cytoplasmic NDRG staining are linked to Gleason grade of PCa. On balance, there seems to be slightly more evidence to support a reduction in higher grades.

There are a number of published studies that describe the association between NDRG1 staining and cancer stage, including PCa. For example, PCa patients with advanced stage had low NDRG1 staining compared to those with localised PCa (Bandyopadhyay *et al.*, 2003). In contrast, two other studies showed membranous and cytoplasmic NDRG1 expression not associated significantly with the clinical stage of PCa. (Caruso *et al.*, 2004; Song *et al.*, 2010). Therefore, there is greater evidence to suggest that alterations in NDRG1 expression are not linked to PCa stages.

Finally, in terms of a link to PCa relapse, a study has found NDRG1 predominately expressed in PCa patients who had a recurrence compared those who had not. However, the authors did not show which localisation of the protein was associated with PCa recurrence (Symes *et al.*, 2013). NDRG1 level was also increased in hepatocellular carcinoma recurrence

compared to non-recurrence (Cheng *et al.*, 2011). Therefore, there are two studies suggesting there might be a link between higher NDRG1 expression and PCa relapse.

Given the evidence, described above, to suggest a link between NDRG1 expression/localisation and PCa it was decided to examine nuclear, membranous and cytoplasmic NDRG1 staining and localisation in normal and malignant prostate tissues, including recurrence and non-recurrence.

The hypothesis used predicted that membranous and cytoplasmic NDRG1 staining will be decreased in PCa compared to NP tissues. Membranous and cytoplasmic NDRG1 staining will be negatively associated with Gleason grade, but not stage, and positively associated with biochemical relapse. These hypotheses are based on the direction of association that was supported by of the most convincing of the studies, but as the evidence is contradictory other possible hypotheses could also have been proposed. There is little evidence regarding the nuclear expression of NDRG1 and PCa, so no hypothesis was proposed, but its expression was examined to see if an association was observed.

3.2.1.3 ATP binding cassette group 2

ATP binding cassette group 2 (ABCG2)/ Breast cancer resistance protein (BCRP), a member of the ABC transporter family, is a transmembrane protein that plays a vital role in promoting proliferation and maintaining the undifferentiated phenotype of SCs (Ding *et al.*, 2010). It is linked to drug-resistance and is thought to be a potential biomarker that can predict clinical progression and prognosis of different kinds of tumours, including breast cancer (Xiang, *et al* 2011) as well as identifying CSCs (Ding *et al*., 2010). Of particular importance for this study, altered expression levels of ABCG2 has been suggested to play a role in PCa progression and relapse (described below and summarised in Table 3.1).

There are a number of publications that have studied ABCG2 protein or mRNA levels in both normal and cancer tissues. Previous studies showed that membranous and cytoplasmic ABCG2 was expressed in breast and oesophageal squamous cell carcinomas (Xiang *et al.*, 2011; Hang *et al.*, 2012). Cytoplasmic ABCG2 expression was significantly decreased in PCa compared to NP and seminal vesicle (Thompson *et al.*, 2008). In contrast, another study showed no significant difference in ABCG2 mRNA levels between normal and malignant prostate samples, using real-time PCR (Guzel *et al.*, 2014). Therefore, there is evidence to suggest that decreased cytoplasmic ABCG2 protein expression, but possibly not mRNA, is linked to PCa formation.

A number of published studies have described the association between ABCG2 expression with tumour grade, clinical stage and biochemical relapse. Increased cytoplasmic ABCG2 staining was observed in PCa tissues with a medium Gleason grade compared to those with either a lower or a higher Gleason grade (Castellon *et al.*, 2012). In other cancers, a previous study found ABCG2 levels in breast cancer significantly associated with clinical stages and lymph node metastasis (Xiang *et al*., 2011*).* In contrast, membranous and cytoplasmic ABCG2 expression was not associated significantly with grades of breast and oesophageal squamous cell carcinomas (Xiang *et al.*, 2011; Hang *et al.*, 2012). There was also no significant difference between ABCG2 levels and oesophageal squamous cell carcinomas stages (Xiang *et al.*, 2011*).* In terms of relapse, ABCG2 mRNA level was significantly increased in PCa relapse compared to non-relapsed (Guzel *et al.*, 2014), and was also higher in CRPC compared to metastatic PCa (Pfeiffer *et al.*, 2011). Therefore, there are conflicting results regarding a link between ABCG2 expression and Gleason grade or clinical stage, but according to previous PCa studies (Pfeiffer *et al.*, 2011; Guzel *et al.*, 2014), its expression might be associated significantly to biochemical relapse.

Given the evidence, described above, to suggest a link between ABCG2 expression and PCa it was decided to examine cytoplasmic ABCG2 staining in normal and malignant prostate tissues, including recurrence and non-recurrence. The hypothesis used predicted that cytoplasmic ABCG2 staining will be decreased in PCa, and will be positively associated with PCa relapse, but not with grade and stage.

3.2.1.4 Aldehyde hydrogenase 1 family member A1

Aldehyde hydrogenase 1 family member A1 (ALDH1A1) represents a marker of CSCs (Kahlert *et al*., 2012; Li *et al*., 2014). This protein is localized in the cytosol of different kinds of human tissues, including prostate (Tomita *et al.*, 2016). It has been reported that ALDH1A1 plays a role to catalyse the oxidation of retinal to retinoic acid that represents a regulator of cell differentiation during the development process (Yoshida *et al*., 1992; Tomita *et al.*, 2016), and is thought to have an essential role in a cancer progression (Li *et al*., 2014). Of particular importance for this study, altered expression levels and localisation of ALDH1A1 has been suggested to play a role in PCa progression, and relapse (described below and summarised in Table 3.1).

There are several publications that studied ALDH1A1 expression/localisation in cancer tissues, including PCa compared to normal tissues. Two studies reported that glandular and

stromal ALDH1A1 staining was observed in cancerous and non-cancerous human tissues such as breast, lung, colon, gastric, liver, pancreas and prostate (Deng *et al.*, 2010; Matsika *et al.*, 2015). In terms of ALDH1A1 glandular nuclear and cytoplasmic expression, previous studies found that cytoplasmic ALDH1A1 staining was increased in cancerous tissues, including PCa compared to non-cancerous tissues (Ting *et al.*, 2009; Kalantari *et al.*, 2017). Nuclear ALDH1A1 expression has not been studied in prostate tissues, however, a study on colon and rectal cancers reported that nuclear ALDH1A1 staining was expressed in only 3% (21/659) colon cancer and only 1% (3/337) rectal cancer (Kahlert *et al.*, 2012). Therefore, there is evidence that suggests alterations in cytoplasmic, but not nuclear, ALDH1A1staining is linked to PCa.

In terms of the association with PCa histopathological and clinical features, increased cytoplasmic ALDH1A1 staining has been suggested to be associated with increasing Gleason grade and advanced stage of PCa (Ting *et al.*, 2009; Kalantari *et al.*, 2017). Another study has reported that there is a significant association between cytoplasmic ALDH1A1 expression and tumours stage, including T and N. In other cancer types, cytoplasmic ALDH1A1 expression was also significantly associated with gastric cancer stages, but not with grades (Li *et al.*, 2014). Less work has focused on nuclear ALDH1A1, but staining was found to be significantly associated with colon cancer grades as well as with lymph node status in rectal cancer (Kahlert *et al.*, 2012). There is currently no study that describes the association between ALDH1A1 expression and PCa relapse, but a study on gastric cancer tissues showed there was a positive association between cytoplasmic ALDH1A1 staining and relapse (Li *et al.*, 2014).

The studies described above focus on the glandular expression of ALDH1A1. However, as mentioned the protein is also expressed in the stromal cells which surround the gland. A link between stromal ALDH1A1 and histopathological parameters of PCa, including Gleason grades, clinical stages and biochemical relapse has not been described before.

Given the evidence, described above, to suggest a link between ALDH1A1 expression/ localisation and PCa (Ting *et al.*, 2009; Kalantari *et al.*, 2017) it was decided to examine cytoplasmic ALDH1A1 in the glands as there was the strongest evidence to support a link between changes in this expression and clinical aspects of PCa. It was also decided to examine nuclear ALDH1A1 in the glands, to provide a comparison to the cytoplasmic expression. Finally, stromal levels were examined in both normal and cancer tissues of the prostate, including recurrence and non- recurrence, as no previous study had looked at the

link between this expression and PCa formation/progression. The hypothesis used predicted that cytoplasmic, but not nuclear, glandular ALDH1A1 will be increased in PCa and positively associated with grade, stage and relapse. No hypothesis was proposed for the stromal expression due to a lack of previous work.

3.2.1.5 Retinoschisin 1

Retinoschisis 1 (RS1) is a protein that is secreted from retinal cells and plays a role in regulating ERK signalling and apoptosis in retinal cells (Tolun *et al.*, 2016; Plössl *et al.*, 2017). X-linked juvenile retinoschisis (XLRS) is a genetic disease that causes vision impairment in young men due to a RS1 mutation (Plössl *et al.*, 2017). Interestingly for this study, previous work in our laboratory suggested that increased RS1 levels might play a role in PCa relapse (described below and summarised in Table 3.1).

According to our knowledge, there is no published study that examines RS1 expression and its association in normal vs. malignant prostate, Gleason grades, clinical stages and biochemical relapse. However, in our laboratory, a PhD student found that nuclear and cytoplasmic RS1 staining was observed in normal and malignant prostate tissues (Sharpe, 2016). The statistical analysis showed that no significant difference in expression of RS1 was observed when comparing malignant prostate tissues to those with a normal nature or between different Gleason grades. However, the cytoplasmic expression of RS1 trended to be higher in relapsed PCa tissues compared to non-relapsed (Sharpe, 2016), but the association was not statistically significant. The main limitation of this study, however, was a small sample size and this result might have been more statistically significant if it had a larger cohort. Therefore, although this protein has not been extensively studied there is some preliminary evidence to suggest that increased cytoplasmic intensity staining of RS1 might be associated with PCa relapse.

Given the preliminary evidence, described above, to suggest a link between RS1 staining and PCa it was decided to examine nuclear and cytoplasmic RS1 staining in a large cohort of normal and malignant prostate tissues, including recurrence and non-recurrence. The hypothesis used, which was based on the previous work from our lab (Sharpe, 2016), predicted that cytoplasmic RS1 staining will be positively associated with PCa relapse, but not with normal vs malignant tissue or between different grades or stages.

Table 3. 1 Candidate protein summary: the type of protein, key publications and the hypothesis for each candidate protein. In some cases, there are contradictory reports on the association between expression of the markers and disease and a range of hypothesis could have been proposed. The hypothesis selected for this study were based on what was felt to be the most convincing data. See text for details.

Name & type of Protein	Key publications	Hypothesis
β-catenin Multifunctional protein involved in adhesion and Wnt signalling.	❖ Membranous β-catenin staining was reduced in PCa and was negatively associated with PCa grade (Kallakury *et al.*, 2001a; Jaggi *et al.*, 2005), but not with recurrence (Kallakury *et al.*, 2001) ❖ Membranous β-catenin staining was increased in PCa tissues with a low grade, whereas, nuclear staining was increased at an advanced stage of PCa (T3-T4) (Aaltomaa *et al.*, 2005). ❖ In localized PCa, nuclear and membranous β-catenin staining is significantly reduced compared to BPH. Reduction of nuclear β-catenin staining was associated significantly with advanced stage and a short biochemical recurrence (Horvath *et al.*, 2005). ❖ Membranous β-catenin expression was reduced in PCa compared to BPH (Bismar *et al.*, 2004; Jaggi *et al.*, 2005). Nuclear β-catenin expression was highly expressed in high grade tissues compared to those with a low grade, whereas, membranous intensity staining was reduced (Jaggi *et al.*, 2005). ❖ β-catenin showed increased levels of nuclear localisation in BPH compared to low and high grades of PCa. Nuclear β-catenin localisation was significantly reduced with increasing grade. There was a reduction of nuclear β- catenin localisation in hormone recurrent PCa compared to non-recurrent, but the data was not significant (Whitaker *et al.*, 2008). ❖ Nuclear localisation of β- catenin in PCa was higher than BPH, but was not associated with Gleason grades and clinical stages, whereas, β- catenin staining intensity was higher in BPH compared to PCa (Ipekci *et al.*, 2015).	❖ Nuclear β-catenin staining will be reduced in PCa compared to NP tissues. ❖ Nuclear β-catenin staining will be negatively associated with Gleason grade, stage and relapse.
NDRG1 Intracellular protein, (cytosolic protein)	❖ Cytoplasmic NDRG1 staining was significantly reduced in high grade PCa compared to low grade. This reduction was significantly associated with advanced stage PCa (Bandyopadhyay *et al*., 2003). ❖ Immunohistochemical study showed that NP tissues had a predominately membranous expression of NDRG1, whereas, decreasing membranous and increasing cytoplasmic expression of NDRGI was significantly associated with increasing Gleason grades. Nuclear NDRG1 staining was only seen in 11 out of 184 PCa cases (Song *et al.*, 2010).	❖ Membranous and cytoplasmic NDRG1 staining will be decreased in PCa compared to NP tissues. ❖ Membranous and cytoplasmic NDRG1 will be negatively

	❖ NDRG1 level was increased in hepatocellular carcinoma recurrence compared to non-recurrence and was significantly associated with tumour stage (Cheng, *et al* 2011). ❖ Reduction nuclear NDRG1 staining is significantly associated with increasing grades and advanced stage of renal cell carcinoma, whereas, membranous expression was not associated with both grades and stages of renal cell carcinoma (Hosoya *et al.*, 2013). ❖ Immunohistochemical study showed increased NDRG1 expression in PCa compared to benign prostate tissues and was positively associated with biochemical relapse, but not with Gleason grade, clinical stage (Symes *et al.*, 2013). ❖ Both NDRG1 protein and mRNA levels were significantly higher in NP cell lines (RWRG1) compared to PCa cell lines (PC3 and LNCap), using IHC, western blot and RT-Q PCR. Cytoplasmic NDRG1 expression was also higher in BPH compared to PCa (Li *et al.*, 2015b).	associated with Gleason grade and positively associated with relapse. Its expression will not be associated with the clinical stage.
ABCG2 A transmembrane transporter protein.	❖ Cytoplasmic ABCG2 expression was significantly reduced in PCa compared to NP and seminal vesicle (Thompson *et al.*, 2008). ❖ ABCG2 mRNA level in CRPC was higher compared with metastatic PCa cases (Pfeiffer *et al.*, 2011). ❖ Cytoplasmic and membranous ABCG2 expression was observed in breast cancer tissues and was associated with clinical stages and lymph node metastasis, whereas, its expression was not associated with grades (Xiang *et al.*, 2011). ❖ ABCG2 was expressed in membranous and cytoplasm of oesophageal squamous cell carcinoma, but was not associated with grades and stages (Hang *et al.*, 2012). ❖ Increased cytoplasmic ABCG2 staining was observed in PCa tissues with a medium Gleason grade compared to those with a low and high Gleason grade (Castellon *et al.*, 2012). ❖ ABCG2 expression showed no significant difference between cancerous and non- cancerous prostate tissues. In PCa, ABCG2 expression was significantly increased in relapse cases compared to non- relapse (Guzel *et al.*, 2014).	❖ Cytoplasmic ABCG2 staining will be decreased in PCa compared to NP tissues. ❖ Cytoplasmic ABCG2 staining will be positively associated with PCa relapse but not with grade and stage.
ALDH1A1 Intracellular enzyme	❖ ALDH1A1was only expressed in the cytoplasm of basal cells of NP, whereas, it was expressed in luminal and neuroendocrine cells of PCa tissues. This cytoplasmic expression was increased in PCa compared to NP tissues and was positively associated with increasing Gleason grades and clinical stages (Ting *et al.*, 2009).	❖ Cytoplasmic, but not nuclear, glandular ALDH1A1stainig will be increased in PCa compared to NP tissues.

	❖ Cytoplasmic ALDH1 expression was higher in tumour tissues such as breast, lung, colon, gastric, liver and pancreas compared to normal tissues. (Deng *et al.*, 2010). ❖ Nuclear and cytoplasmic ALDH1A1 expression was observed in normal colon, and a few cases showed stromal expression of ALDH1A1. Colon and rectal cancer tissues had a cytoplasmic expression of ALDH1A, but was not associated with all histopathological parameter of the colon and rectal cancers except lymph node status of rectal cancer. In addition, 3% (21/659) colon cancer and only 1% (3/337) rectal cancer tissues showed positive nuclear ALDH1A1 staining. Nuclear ALDH1A1 staining was significantly associated with colon cancer grades and lymph node status (Kahlert *et al.*, 2012). ❖ Half of the gastric cancer samples showed cytoplasmic ALDH1A1 expression. Increasing cytoplasmic expression of ALDH1A1 was significantly associated with advanced stages, and relapse, but not with grades. (Li *et al.*, 2014). ❖ An IHC study showed cytoplasmic ALDH1A1 expression in about half of PCa samples (67/142). This study reported that there was a patch of positive ALDH1A1 in the stroma of PCa. Cytoplasmic ALDH1A1 expression showed a significant association with PCa stages, including tumours stage (T) and lymph node invasion (N) (Matsika *et al*., 2015). ❖ Cytoplasmic ALDH1A1 expression was significantly increased in PCa compared to BPH and PIN. Cytoplasmic ALDH1A1 was significantly associated with Gleason score and primary tumours stage and lymph node status (Kalantari *et al*., 2017).	❖ Cytoplasmic, but not nuclear, glandular ALDH1A1 staining will be positively associated with grades, stages and relapse.
RS1 A secreted protein	❖ Nucleus and cytoplasm RS1 staining showed no significant difference between cancerous and non- cancerous prostate tissues. Although there was no significant difference between nuclear and cytoplasm RS1 staining and Gleason score and biochemical relapse. However, cytoplasmic RS1 staining was trended to be higher in PCa relapsed patients compared to a non-relapsed (Sharpe, 2016).	❖ Cytoplasmic RS1 will not be altered in PCa compared to NP tissue. ❖ Cytoplasmic RS1 will be positively associated with PCa relapse, but not with grade and stage.

3.2.2 Testing prostate tissue morphology using Haematoxylin and Eosin staining

Having chosen the candidate proteins, the next step is to assess their expression using IHC. Before this could be carried out the morphology of the tissue from RUH needed to be confirmed by staining it with H&E. Both normal and malignant prostate tissue samples were stained with H&E (Figure 3.1). NP tissues stained with H&E staining showed prostate glands which consist of different cell types, including basal (Figure 3.1A, blue arrows) and luminal cells (Figure 3.1 A, red arrow) surrounded by fibromuscular stroma (Figure 3.1 A, black arrow). In contrast, PCa tissues had a disruption of the normal structure of prostate glands which showed increased luminal cells (Figure 3.1 B, red arrows) and the absence of basal cells. These glands were also surrounding by fibromuscular stroma (Figure 3.1 B, black arrow). This H&E result confirms that the morphology was as expected and so IHC could then be carried out in the next section.

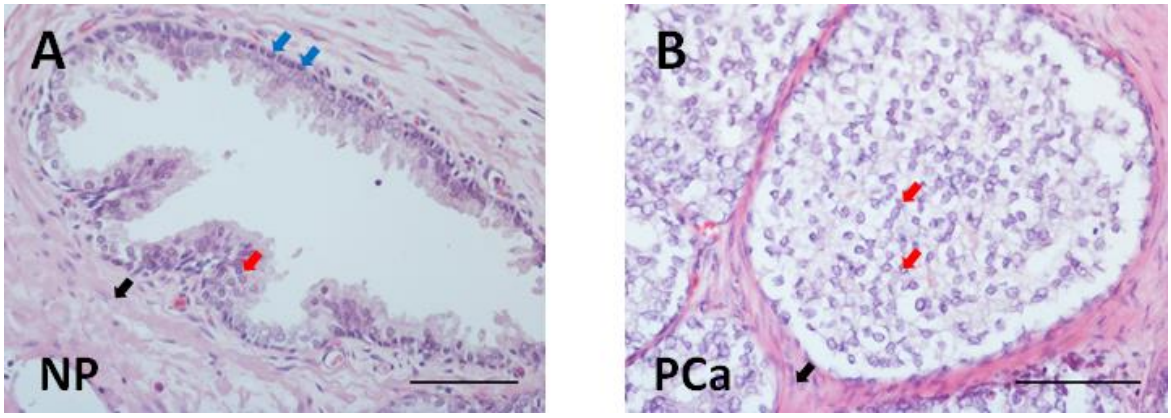

Figure 3. 1 Checking the histological structure of the prostate tissue from the Bath cohort. (A) The NP stained with H&E showed prostate gland cells, including basal cells (Blue arrows) and luminal cells (Red arrow) surrounding by fibromuscular stroma (Black arrow). (B) PCa consists of prostate gland cells such as luminal cells (Red arrows) and fibromuscular stroma (Black arrow). The tissue organisation was as expected for the normal and malignant prostate. Scale bar=100μm.

3.2.3 Establishing IHC protocols for the proteins of interest

The next steps in establishing IHC protocols for the proteins of interest was to select antibodies and then test them with different antigen retrieval protocols to establish if they produced staining patterns matching those expected from previous studies reported in the literature. Five different antibodies, one against each of β-catenin, NDRG1, ABCG2, ALDH1A1 and RS1 were selected in this study (see Chapter 2.1.2), using the CiteAb website (https://www.citeab.com). The antibodies were selected based on the following criteria: that

they had been used and validated previously for IHC staining, they reacted with human tissues and were produced by a well-known company.

Two different types of antigen retrieval, treatment with citrate (pH6) and Tris/ EDTA (pH9) buffers at 90 ^{0}C, using a water bath, were tested to see which provided the best staining for each antibody. β-catenin had membranous, cytoplasmic and nuclear staining, as expected, following both antigen retrieval methods (Figure 3.2). However, a citrate buffer was chosen because clearer nuclear and cytoplasmic staining for β-catenin was observed. IHC staining showed membranous, cytoplasmic and nuclear NDRG1 staining in both buffers. However, the citrate buffer showed clearer nuclear and cytoplasmic NDRG1 staining compared to Tris/ EDTA buffer (Figure 3.2). NDRG1 staining was expected to be in the membrane, cytoplasmic and nucleus of cells. The citrate buffer was also the best choice for ALDH1A1 in this study. This is because IHC results after using citrate buffer antigen retrieval showed clearer nuclear and cytoplasmic glandular and stromal ALDH1A1 staining compared with Tris/ EDTA buffer results, where the samples appeared over stained (Figure 3.2). Nuclear and cytoplasmic ALDH1A1staining matched the expected staining pattern for this protein. ABCG2 had membranous, cytoplasmic and nuclear staining in both buffers. However, IHC results using Tris/ EDTA buffer showed clearer membranous ABCG2 staining and less nuclear staining compared to retrieval using citrate (Figure 3.2). ABCG2 is a transmembrane transporter protein and is not expected to be in the nucleus of the cells, so this study used the Tris/EDTA buffer for antigen retrieval for ABCG2. Finally, IHC results with both buffers showed nuclear and cytoplasmic RS1 staining in prostate tissues. However, the citrate results were clearer than Tris/ EDTA results (Figure 3.2). This pattern of staining matched that observed previously in our laboratory, although as a secreted protein RS1 would not be expected to be found in the nucleus, so this proportion of the staining might be background.

In summary, after testing antigen retrieval methods, the citrate buffer (pH6) was preferred for all candidate biomarkers (β-catenin, NDRG1, ALDH1A1 and RS1), except for ABCG2, where Tris/ EDTA was selected and all the antibodies were felt to be suitable for use as the staining patterns were consistent with that described previously in the literature.

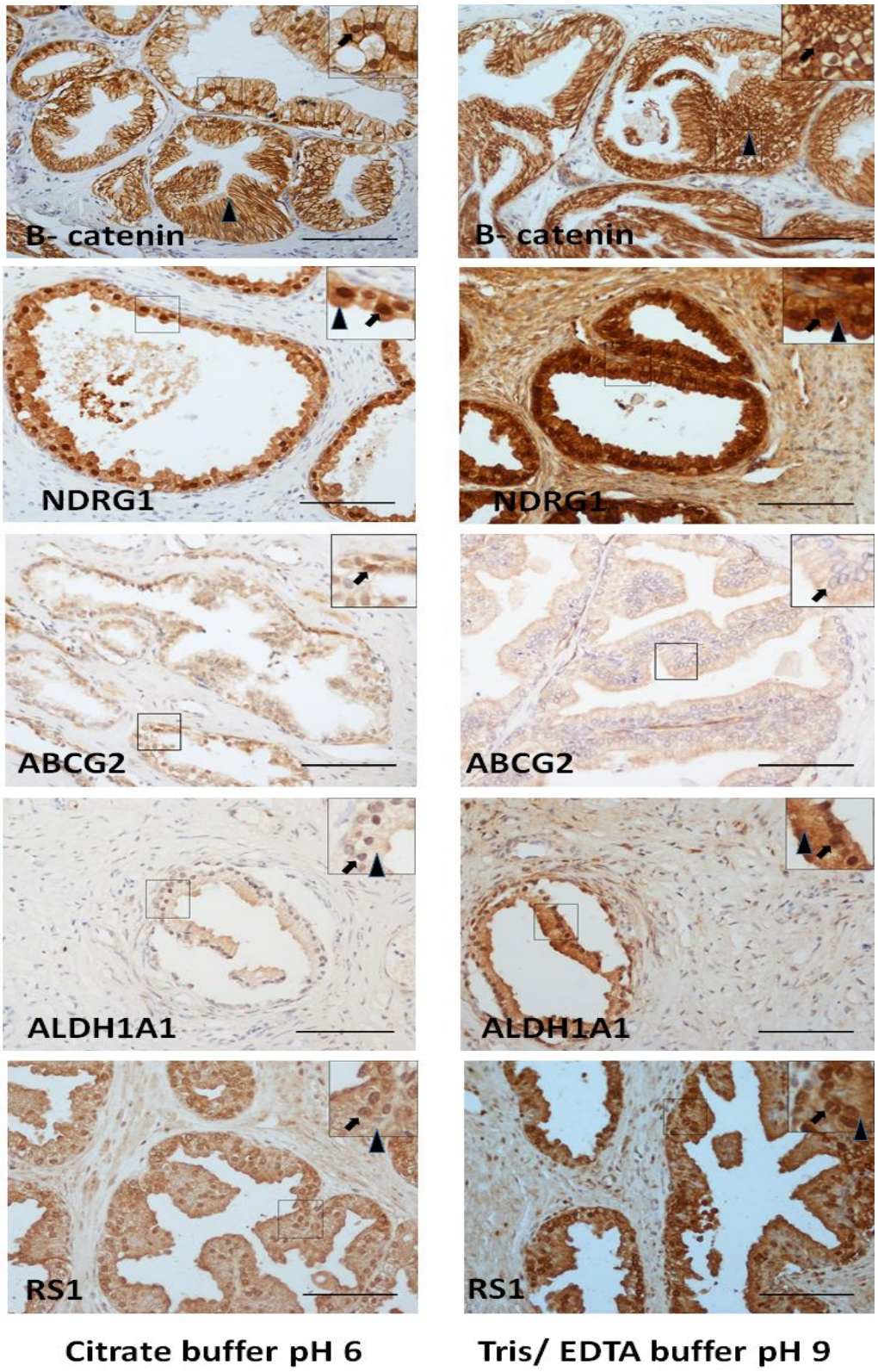

Figure 3. 2 Expression of the potential biomarkers after the different antigen retrieval methods. (A) Expression of these potential biomarkers after using citrate buffer pH 6. (B) Expression of these potential biomarkers after using Tris/EDTA buffer pH 9. All the potential biomarkers showed a clearer result with citrate buffer, except ABCG2 that showed clearer staining in Tris/EDTA. Scale bar=100μm.

3.2.4 Structure of the Bath cohort

The Bath cohort consists of 34 PCa paraffin-embedded blocks, including those from patients who had and had not undergone recurrence, and five NP blocks. The samples were obtained from the histopathological laboratories of the RUH, Bath, UK. The samples were collected between 1997 and 2018. This study also used normal testis, kidney and liver tissues as positive controls. A summary of the Bath cohort clinical data is shown in (Table 3.2). This cohort of samples was then stained using antibodies against the different potential biomarkers.

Table 3. 2 Clinical data of the Bath cohort prostate samples.

The Bath Clinical data		**Number**	**%**
Number of samples	Normal	5	
	Malignant	34	
Age range	Normal	N/A	
	Malignant	58-81	
Primary Gleason Grade	Grade 3	15	44.1
	Grade4	16	47
	N/A	3	8.9
T category	T1-T2	19	55.9
	T3-T4	9	26.5
	N/A	6	17.6
N category	N0	24	70.6
	N1	3	8.8
	N/A	7	20.6
M category	M0	20	58.8
	M1	3	8.8
	N/A	11	32.4
Biochemical recurrence status (at 5 years)	Non-Recurrent	13	38.2
	Recurrent	19	55.9
	N/A	2	5.9

3.2.5 β-catenin

To test the hypothesis that nuclear β-catenin staining will be decreased in PCa and negatively associated with primary Gleason grade, clinical stage and relapse; IHC was carried out with the Bath cohort prostate samples.

3.2.5.1 Immunohistochemical staining of β-catenin in the normal and malignant Bath prostate tissues

β-catenin staining was carried out using IHC on prostate samples from the Bath cohort. In NP tissues, IHC staining showed nuclear β-catenin staining which ranged from strong (Figure 3.3 A, arrow), moderate (Figure 3.3 B, arrow) and weak in some cases. Nuclear β-catenin staining was also observed in PCa tissues and the intensity of signal varied widely, from strong (Figure 3.3 D, arrow) to weak (Figure 3.3 F, arrow). Membranous β-catenin staining was also observed in the Bath prostate tissue samples with variable levels of staining, ranging from strong (Figure 3.3 D, red arrowhead), moderate (Figure 3.3 C, red arrowhead), weak (Figure 3.3 E, red arrowhead) and negative (Figure 3.3 B, red arrowhead).

In addition to the nuclear and membranous staining, normal and malignant prostate tissues had cytoplasmic β-catenin staining, with variable levels of staining between cases, ranging from strong (Figure 3.3 D, arrowhead), moderate (Figure 3.3 A, arrowhead) and weak (Figure 3.3 B, C, E & F, arrowheads). β-catenin is expressed in Sertoli, spermatid and spermatozoa cells of normal testis tissues (Lee *et al.*, 2005) and so this study used the normal testis as a positive control for β-catenin and as expected IHC staining showed nuclear β-catenin staining in the spermatid and spermatozoa cells of testis tissues (Figure 3.3 G, arrow). A negative control, with no primary antibody, showed no significant background staining in prostate tissue (Figure 3.3 H, arrow).

3.2.5.2 Association between β-catenin immunostaining and histopathological parameters of prostate cancer in the Bath cohort

Having carried out IHC staining on normal and malignant prostate samples from the Bath cohort, the nuclear and cytoplasmic β-catenin staining was then quantified using H and proportion and intensity 1 scores, respectively. The range of nuclear and cytoplasmic β-catenin scores varied between 0 to 110 and 0 to 7, respectively (Figure 3.4). The potential association between β-catenin IHC results and histopathological parameters of PCa was then analysed and is described below.

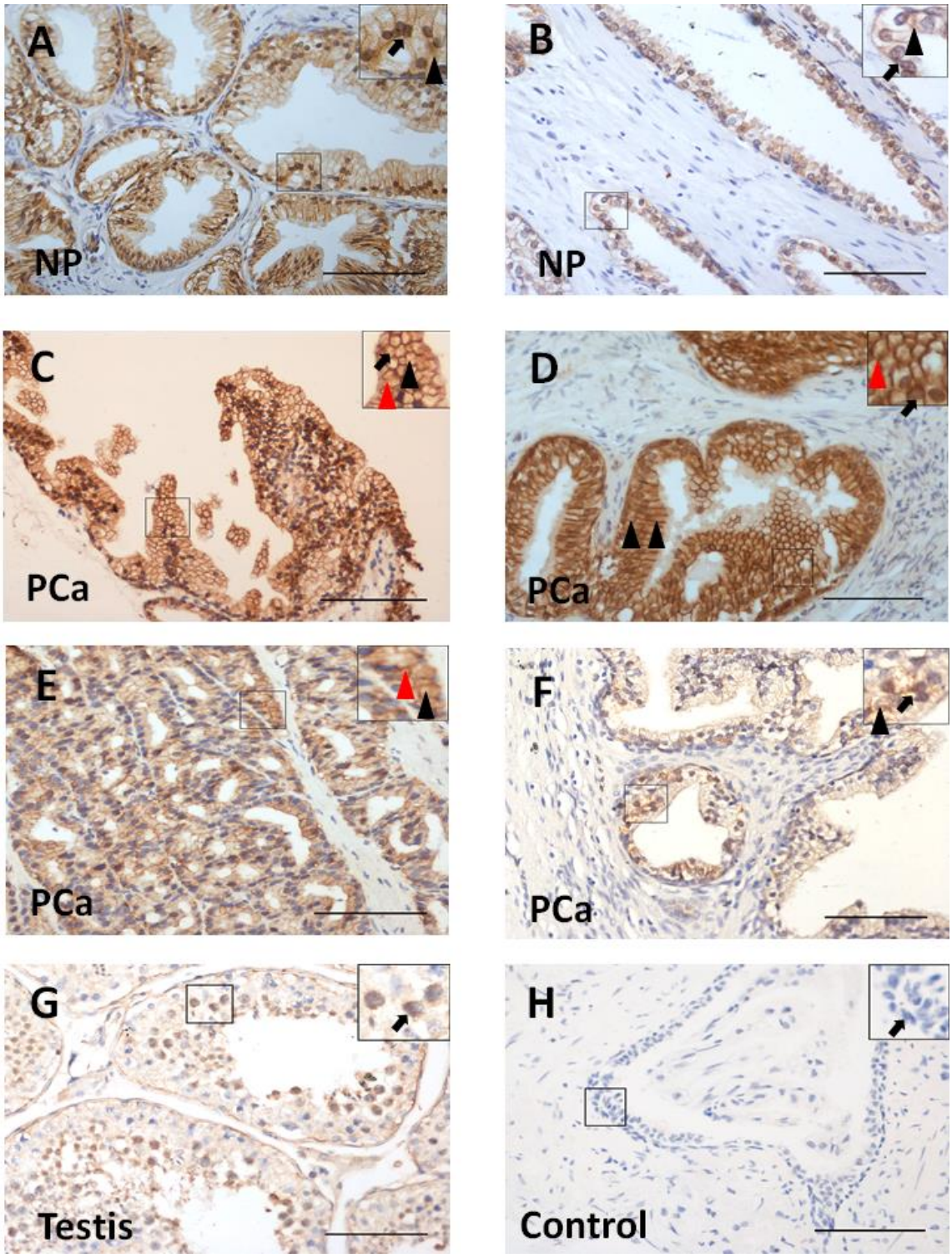

Figure 3. 3 β-catenin staining in samples from the Bath cohort. β-catenin was stained heterogeneously in both normal and malignant tissues of the prostate. (A) Strong nuclear (Black arrow) and moderate cytoplasmic (Black arrowhead) β-catenin staining in NP. (B) Weak to moderate nuclear (Black arrow) and cytoplasmic (Black arrowhead) β-catenin staining in NP. (C) Weak cytoplasmic (Black arrowhead) and moderate membranous (Black arrow) β-catenin staining in PCa. (D) Strong nuclear (Black arrow), membranous (Red arrowhead) and cytoplasmic (Black arrowheads) β-catenin staining in PCa. (E) Weak membranous (Red arrowhead) and cytoplasmic (Black arrowhead) β-catenin staining in PCa. (F) Moderate nuclear (Black arrow) and weak cytoplasmic (Black arrowhead) β-catenin staining in PCa. (G) Nuclear β-catenin staining (Black arrowhead) in testis tissue. (H) Negative control (no primary antibody added) was free background staining (Black arrow) in PCa. Scale bars=100μm.

The first analysis looked at the β-catenin staining in normal vs. malignant prostate tissues. Quantification of the IHC staining showed reduced nuclear β-catenin staining significantly in PCa compared to NP (p= 0.0066) (Figure 3.4 A & Table 3.3). There appeared to be a trend towards decreased cytoplasmic β-catenin scores in PCa compared to NP (Figure 3.4 B& Table 3.3), although this was not significant (p=0.1923). Nuclear and cytoplasmic β-catenin staining was also reduced significantly in PCa patients with a primary Gleason grade 4 compared to those with a Gleason grade 3 (p= 0.0447, 0.0127, respectively) (Figure 3.4 C&D & Table 3.3). In contrast, nuclear and cytoplasmic β-catenin staining was not associated significantly with clinical stage T (T1-2 vs. T3-4) (p=0.2525, 0.9288, respectively) and biochemical relapse (p= 0.2808, 0.5135 respectively) (Table 3.3).

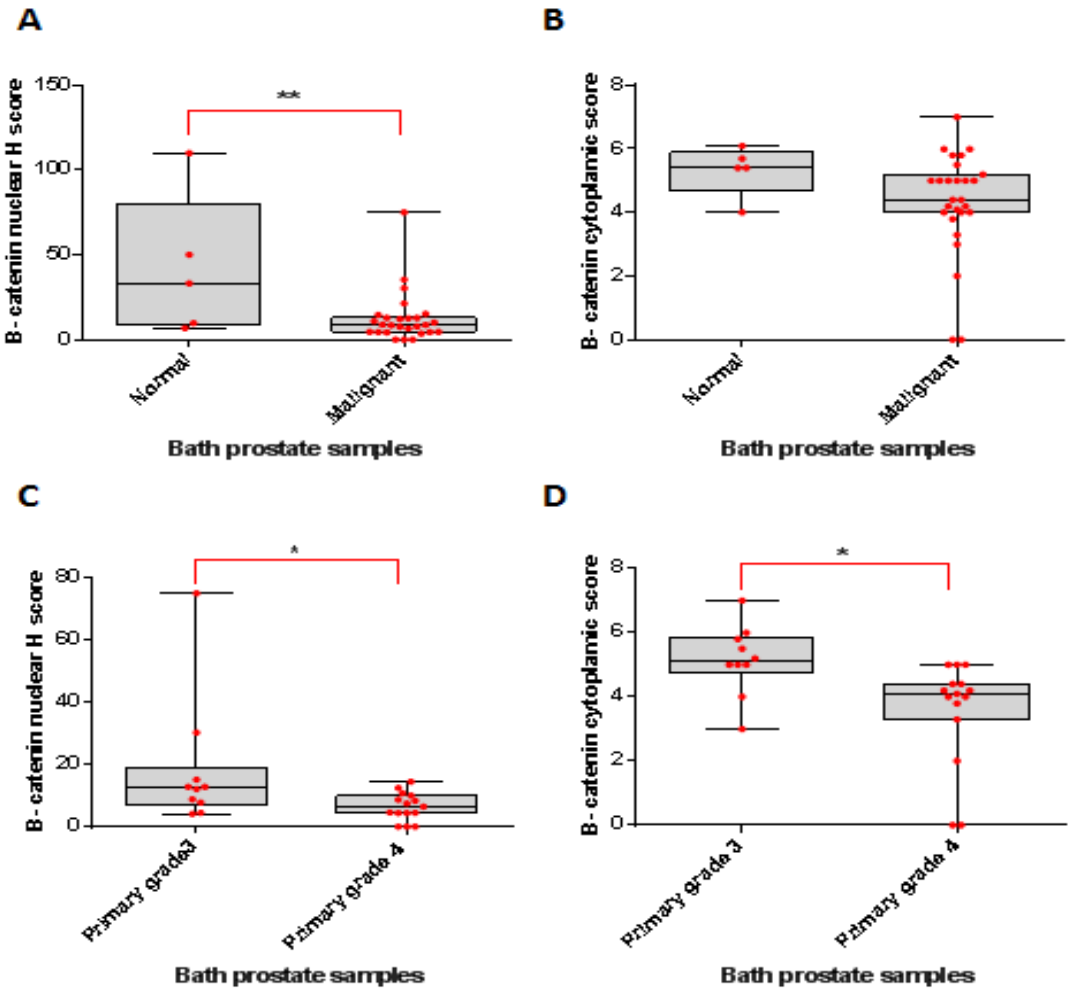

Figure 3. 4 Quantification of nuclear and cytoplasmic β-catenin staining in both normal and malignant Bath prostate tissues. IHC staining of β-catenin was quantified in the Bath cohort using H and the proportion and intensity 1 score for nuclear and cytoplasmic IHC staining. (A) Nuclear β-catenin staining was significantly reduced in PCa compared with NP tissues (p=0.0066). (B) Cytoplasmic β-catenin staining was reduced but not significantly in PCa compared with the NP (P= 0.1923). (C) Nuclear β-catenin staining was significantly reduced in primary Gleason grade 4 compared with grade 3 (p= 0.0447). (D) Cytoplasmic β-catenin staining was also significantly reduced in primary Gleason grade 4 compared to grade 3 (p= 0.0127). The mean of five random fields was taken per prostate sample. Statistical significance was determined with unpaired T-test for each set of conditions. NP (n=5), PCa (n=27), grade 3 (n=10) and grade 4 (n=15).

Table 3. 3 Nuclear and cytoplasmic β-catenin staining results with clinical data.

Comparison	Nuclear β-catenin staining		Cytoplasmic β-catenin staining	
	Results	**p. value**	**Results**	**p. value**
Normal vs malignant	Lower in malignant	0.0066	No statistically significant difference	0.1923
Primary Gleason grades (3 & 4)	Lower in high grade	0.0447	Lower in high grade	0.0127
Clinical Stage (T)	No statistically significant difference	0.2525	No statistically significant difference	0.9288
5 years Biochemical recurrence	No statistically significant difference	0. 2808	No statistically significant difference	0.5135

In summary, nuclear β-catenin staining was reduced in malignant prostate tissues compared to those with a normal nature, whereas, cytoplasmic β-catenin was not associated significantly with PCa. Nuclear and cytoplasmic β-catenin staining was negatively associated with increasing primary Gleason grade, but not with clinical stage T and biochemical recurrence.

3.2.6 NDRG1

To test the hypothesis that membranous and cytoplasmic NDRG1 staining will be decreased in PCa and will be negatively associated with increasing primary Gleason grade and will be positively associated with relapse, but not with the stage; IHC was carried out with the Bath prostate samples.

3.3.6.1 Immunohistochemical staining of NDRG1 in the normal and malignant Bath prostate tissues

IHC staining showed that membranous, cytoplasmic and nuclear NDRG1 staining was detected in the Bath prostate tissues. Membranous NDRG1 was expressed predominantly in NP tissues (Figure 3.5 A, red arrowhead). In contrast, only one case of PCa showed predominant membranous staining (Figure 3.5 F, red arrowhead). NP tissues had cytoplasmic NDRG1 staining with variable levels of staining patterns, ranging from strong (Figure 3.5 A, black arrowhead) to weak (Figure 3.5 B, black arrowhead). Cytoplasmic

NDRG1 staining was found in PCa tissues with different level of staining, ranging from strong (Figure 3.5 C, black arrowhead), moderate (Figure 3.5 F, black arrowhead) and weak (Figure 3.5 E, arrowhead). In addition, the Bath prostate tissues showed nuclear NDGR1 staining with a variable level of staining, ranging from strong (Figure 3.5 B&C, arrows), moderate (Figure 3.5 D, arrow), weak (Figure 3.5 E, arrow) and negative (Figure 3.5, F, arrow). NDRG1 is expressed in kidney tissues (Lachat *et al.*, 2002) and so this IHC study used normal kidney tissue as a positive control for NDRG1 and as expected cytoplasmic NDRG1 staining was observed in kidney tissue (Figure 3.5, G arrowhead). A negative control, no primary antibody, was clear of background staining in prostate tissue (Figure 3.5 H, arrow).

3.3.6.2 Association between NDRG1 immunostaining and histopathological parameters of prostate cancer in the Bath cohort

3.3.6.2.1 NDRG1 localisation

The analysis of NDRG1 staining started by using a different approach to the other markers. The predominant localisation, rather than the amount of staining, was quantified in normal and malignant prostate tissues from the Bath cohort. The localisation was divided into three different groups: predominant membranous, predominant nucleocytoplasmic and negative and each sample scored for its most common localisation. This was carried out to match the previous analysis of this protein (Caruso *et al.*, 2004).

The first analysis looked at the NDRG1 localisation in normal vs. malignant prostate tissues. Predominant membranous NDRG was shown in 40% of NP tissues, whereas, only 4% of PCa had predominant membranous (Figure 3.6 A). In contrast, nucleocytoplasmic NDRG1 was expressed predominantly in 60 % of NP and 92 % of PCa tissues (Figure 3.6 A). Only one case of PCa showed no significant staining for NDRG1 (Figure 3.6 A). Quantification of the IHC staining showed that there was a significant difference in NDRG1 localisation (membranous vs. nucleocytoplasmic) between normal and malignant prostate tissues ($p=0.0167$). There was no significant association between NDRG1 localisation and PCa clinical features, including primary Gleason grade, clinical stage T and relapse (Figure 3.6 B, C, & D). Frequency tests, however, showed that predominant nucleocytoplasmic NDRG1 was higher with increasing primary Gleason grade. All PCa tissues with a grade 4 showed predominant nucleocytoplasmic NDRG1 staining. In contrast, nucleocytoplasmic NDRG1 was observed in 80% of PCa tissues with a grade 3 (Figure 3.6 B).

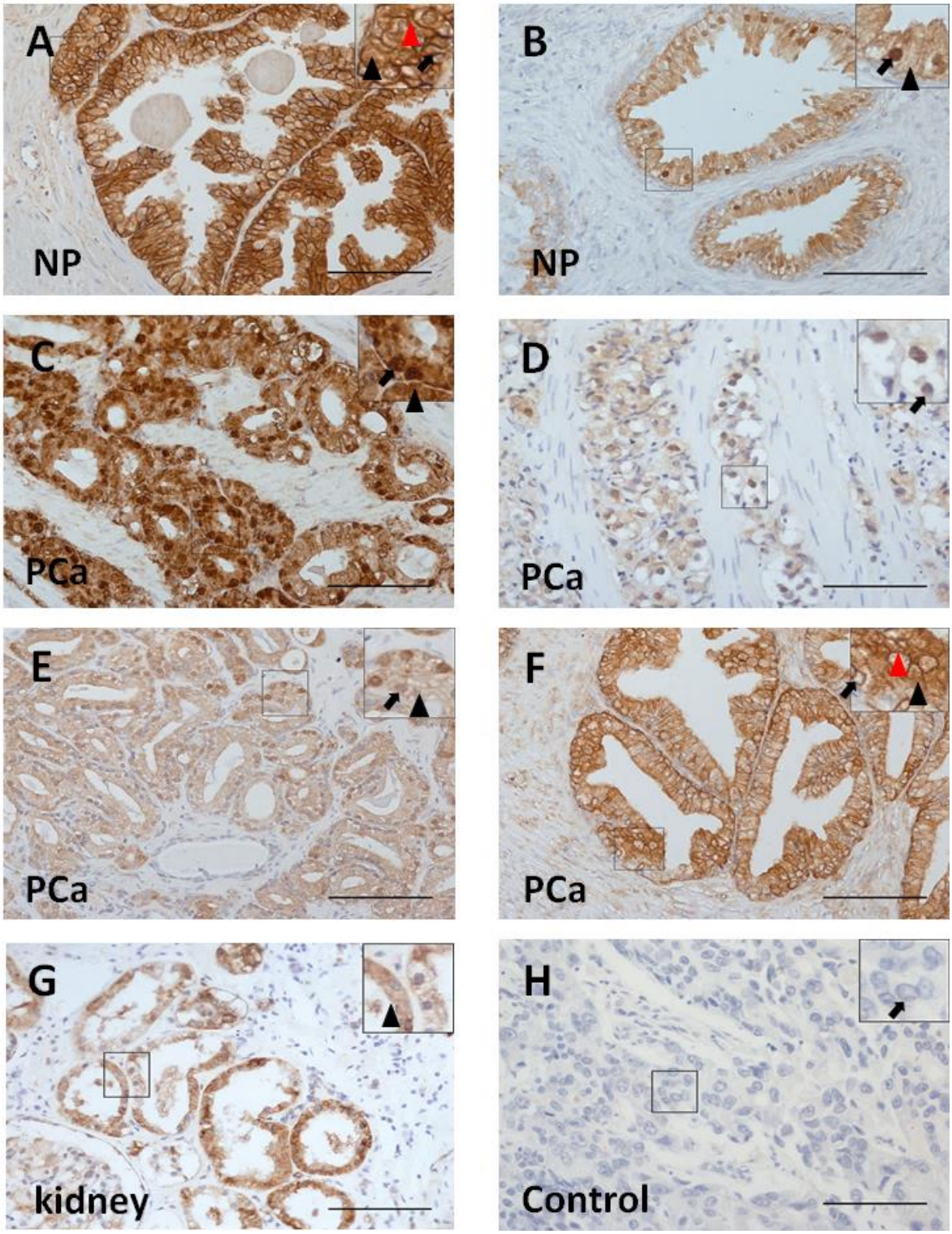

Figure 3. 5 NDRG1 staining in samples from the Bath cohort. NDRG1 was stained heterogeneously in both normal and malignant tissues of the prostate. (A) Predominant membranous (red arrowhead) and cytoplasmic NDRG1 staining (Black arrowhead) in NP. (B) Strong nuclear (Black arrow) and weak cytoplasmic (Black arrowhead) NDRG1 staining in NP. (C) Strong nuclear (Black arrow) and cytoplasmic (Black arrowhead) NDRG1 staining in PCa. (D) Moderate nuclear (Black arrow) NDRG1 staining in PCa. (E) Weak nuclear (Black arrow) and cytoplasmic (Black arrowhead) NDRG1 staining in PCa. (F) Moderate Membranous (Red arrowhead) and cytoplasmic (Black arrowhead) with negative nuclear NDRG1 staining in PCa. (G) Cytoplasmic NDRG1 staining (Black arrowhead) in kidney tissue. (H) The negative control (no primary antibody) showed no staining (Black arrow) in prostate tissue. Scale bars=100µm.

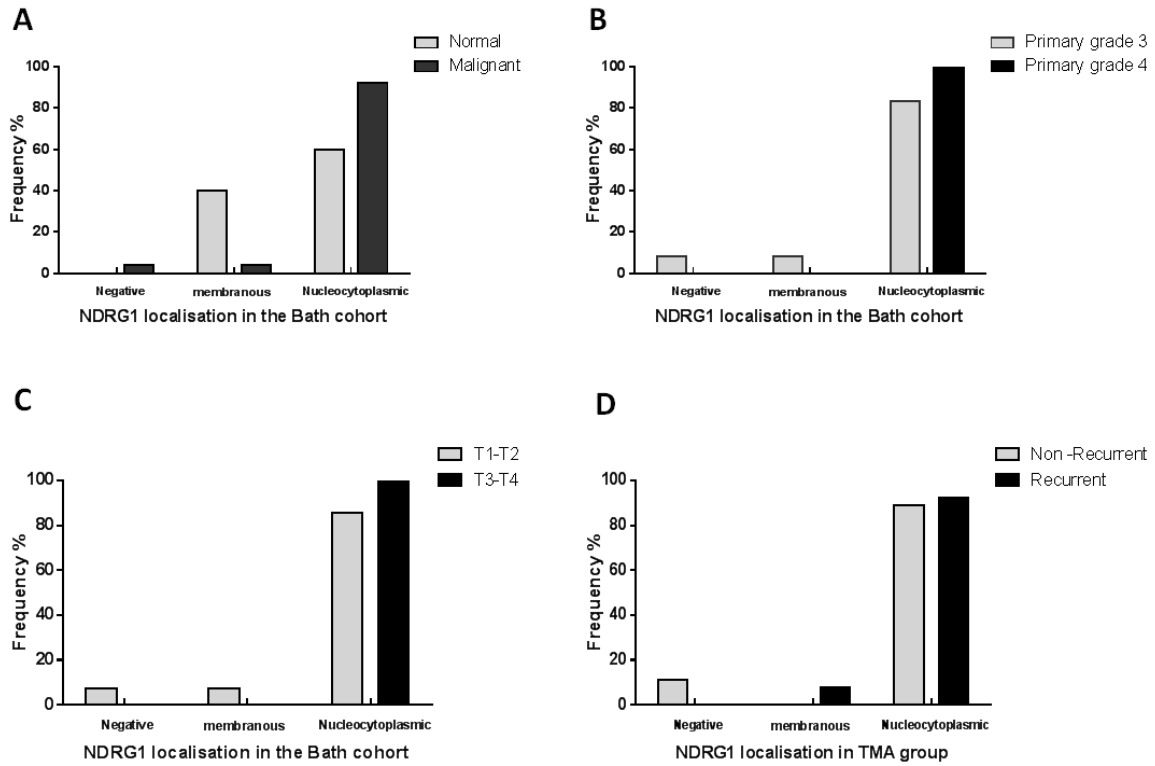

Figure 3. 6 Localisation of NDRG1 in the Bath PCa samples. IHC staining of NDRG1 localisation was quantified in the TMA group using the localisation 1 score. (A) Predominant nucleocytoplasmic was positively associated with PCa (P=0.0167). (B) NDRG1 localisation was not associated significantly with primary Gleason grade p= (0.2186). (C) There was no clear difference between NDRG1 localisation and clinical stage T (p=0.3944). (D) There was no significant difference between NDRG1 localisation and PCa recurrence (p=0.4632). The mean of five random fields was taken per prostate sample. Statistical significance was determined with a Chi-Square and frequency tests for each set of conditions. NP (n=5), PCa (n=25), grade 3 (n=10), grade 4 (n=15), T1-2 (n=14), T3-4 (n=9), non-Recurrence (n=9) and recurrence (n=13).

3.2.6.2.2 NDRG1 staining

The analysis of localisation suggested a link between NDRG1 staining and clinical parameters, so the levels of nuclear and cytoplasmic NDRG1 staining was then quantified using the proportion and intensity 2 scores. The range of nuclear and cytoplasmic anti-NDRG1 scores varied between 0-6.5. The potential association between NDRG1 IHC results and histopathological parameters of PCa was then analysed and is described below (Table 3.4). Nuclear and cytoplasmic NDRG1 staining showed no significant difference between normal vs. malignant prostate tissues (P= 0.3051 & 0.0963, respectively) (Table 3.4) as well as other PCa histopathological features (Table 3.4).

Table 3. 4 Nuclear and cytoplasmic NDRG1 staining results with clinical data

Comparison	Nuclear NDRG1 staining		Cytoplasmic NDRG1 staining	
	Results	**p. value**	**Results**	**p. value**
Normal vs malignant	No statistically significant difference	0.3051	No statistically significant difference	0.0963
Primary Gleason grades (3 & 4)	No statistically significant difference	0.2534	No statistically significant difference	0.8356
Clinical Stage (T)	No statistically significant difference	0.4409	No statistically significant difference	0.8317
5 years Biochemical recurrence	No statistically significant difference	0.6544	No statistically significant difference	0.8925

In summary, there was a significant difference in NDRG1 localisation between normal and malignant prostate, but was not associated with Gleason grade, stage and relapse. Nuclear and cytoplasmic NDRG1 staining was also not associated significantly with PCa and other clinical features of PCa.

3.2.7 ABCG2

To test the hypothesis that cytoplasmic ABCG2 staining will be decreased in PCa and will be positively associated with biochemical relapse, but not with Gleason grade and stage; IHC was carried out with the Bath prostate tissue samples.

3.2.7.1 Immunohistochemical staining of ABCG2 in the normal and malignant Bath prostate samples

The IHC staining showed that normal and malignant prostate tissues had membranous ABCG2 staining with variable levels of staining, ranging from strong and widespread (Figure 3.7 C, arrow) to moderate and scattered (Figure 3.7 A, arrow) or negative (Figure 3.7 E, arrow). Nuclear ABCG2 staining was also observed in the Bath prostate tissues with different levels of staining, ranging from strong (Figure 3.7 B, arrow) to weak (Figure 3.7 F, arrow) or negative (Figure 3.7 D, arrow). ABCG2 would not be predicted to be expressed in the nuclei of normal and malignant prostate tissues because it's a transmembrane transporter protein. This suggests that this staining might well be background, however, it was decided to quantify it as it might act as an internal control that would not be expected to be linked to any disease progression. Normal and malignant prostate tissue samples also had cytoplasmic

ABCG2 staining which, unlike the nuclear staining, is expected. The intensity of signal varied widely, ranging from strong and widespread (Figure 3.7 C, arrowhead), moderate (Figure 3.7 D, arrowheads), weak (Figure 3.7 A, B& E, arrowheads), and negative (Figure 3.7 F, arrowhead). ABCG2 is expressed in the membrane and cytoplasm of hepatocyte cells (Vander Borght *et al.*, 2006) and so this study used normal liver tissues as a positive control for ABCG2 and the IHC staining showed membranous (Figure 3.7 G, arrow) and cytoplasmic (Figure 3.7 G, arrowheads) ABCG2 staining in the hepatocyte cells. A negative control (no primary antibody) showed no significant background staining in prostate tissue (Figure 3.7 H, arrow).

3.2.7.2 Association between ABCG2 immunohistochemistry and histopathological parameters of prostate cancer in the Bath cohort

In order to evaluate if there was an association between ABCG2 staining in normal vs. malignant prostate tissues and/or other PCa histopathological features, nuclear and cytoplasmic ABCG2 staining was quantified using the proportion and intensity 1 scores and then compared to histopathological and clinical parameters of PCa using the clinical data available for the Bath cohort. The range of nuclear and cytoplasmic ABCG2 varied between 0 - 2.8 and 0 - 7, respectively (Figure 3.8). The potential association between ABCG2 IHC results and other PCa histopathological parameters was then analysed and is described below.

The statistical analysis looked at the expression of ABCG2 in normal vs. malignant prostate tissues. Quantification of the IHC staining showed no significant difference in nuclear ABCG2 scores between NP tissues compared to those with malignancies (p=0.7873) (Figure 3.8 A Table 3.5) or relapsed vs non relapsed PCa (p = 0.8106) (Figure 3.8 G & Table 3.5). In contrast, nuclear ABCG2 staining was positively associated with increasing primary Gleason grade and clinical stage T (p = 0.0221& 0.0337 respectively) (Figure 3.8 C, E & Table 3.8).

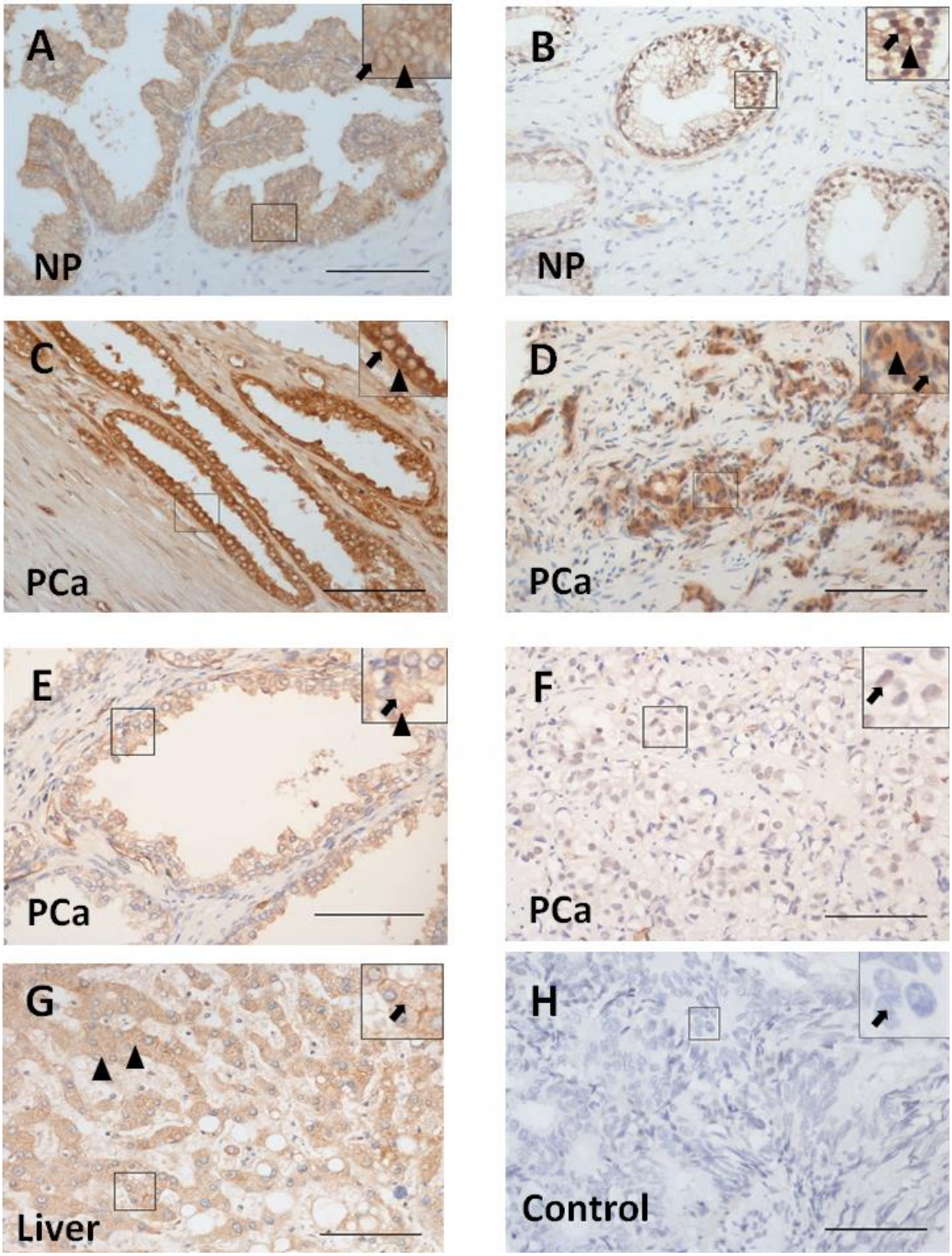

Figure 3. 7 ABCG2 staining in samples from the Bath cohort. ABCG2 was stained heterogeneously in both normal and malignant tissues of the prostate. (A) Moderate membranous (Black arrow) and weak cytoplasmic (Black arrowhead) ABCG2 staining in NP. (B) Strong nuclear (Black arrow) and weak cytoplasmic (Black arrowhead) ABCG2 staining in NP. (C) Strong membranous (Black arrow) and cytoplasmic (Black arrowhead) ABCG2 staining in PCa. (D) Moderate cytoplasmic (Black arrowhead) and negative nuclear (Black arrow) ABCG2 staining in PCa. (E) Weak cytoplasmic (Black arrowhead) with negative nuclear ABCG2 staining in PCa. (F) Weak nuclear (Black arrow) and negative cytoplasmic ABCG2 staining in PCa. (G) Membranous (Black arrow) and cytoplasmic (Black arrowheads) ABCG2 staining in hepatocyte cells. (H) Negative control (no primary antibody added) showed no background staining (Black arrow) in PCa. Scale bars=100µm.

Cytoplasmic ABCG2 staining was significantly reduced in PCa compared to NP tissues (p= 0.048) (Figure 3.8 B & Table 3.5). Cytoplasmic ABCG2 staining was negatively associated with increasing primary Gleason grade, but the result was not significant (p=0.1199) (Figure 3.8 D & Table 3.5). There was no significant association between cytoplasmic ABCG2 staining and clinical stage (p=0.327) (Figure 3.8 F &Table 3.5). In contrast, cytoplasmic ABCG2 staining was reduced significantly in PCa recurrent patients compared to those with a non-recurrent (p=0.0141) (Figure 3.8 H & Table 3.5).

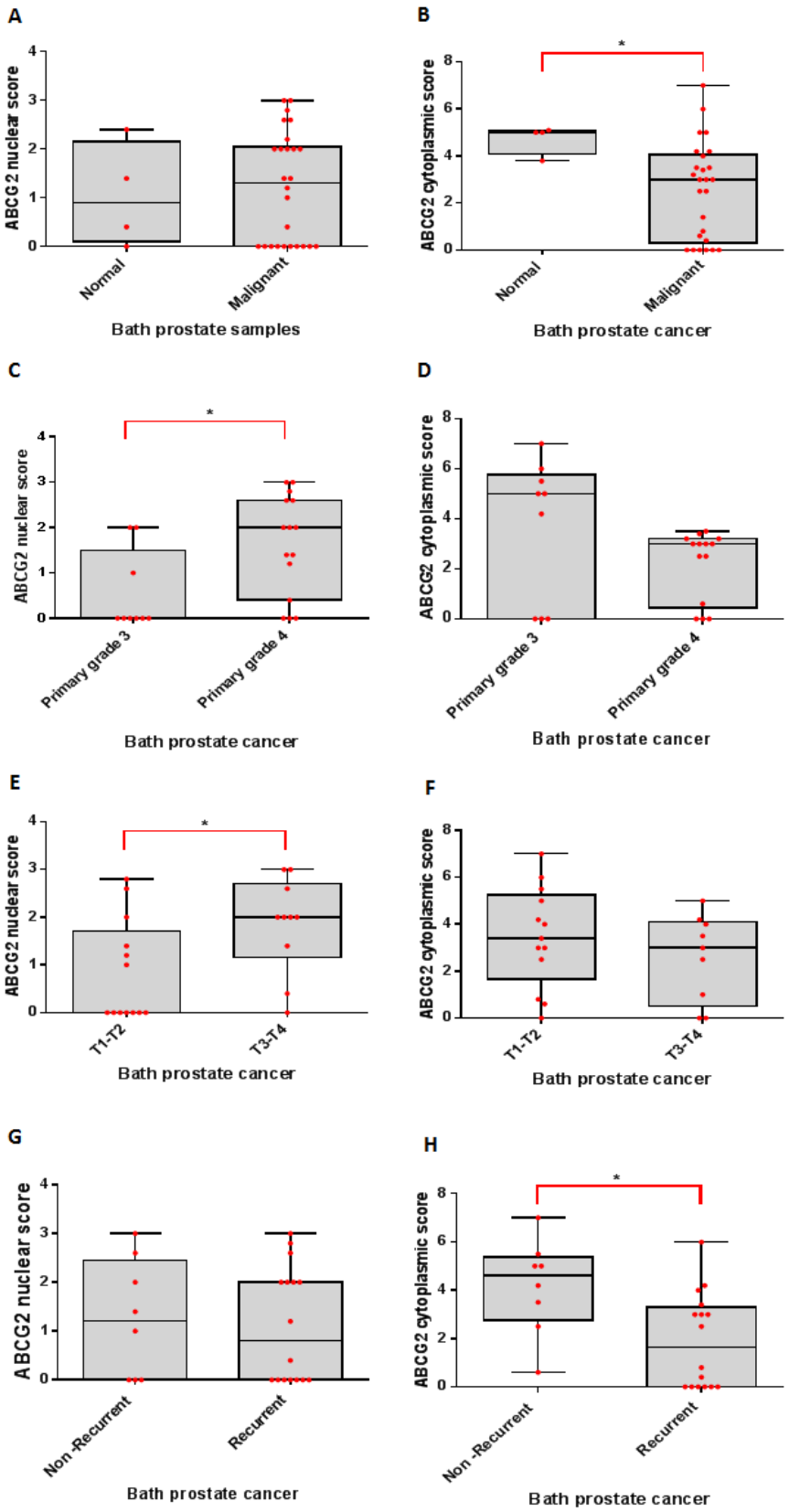

Figure 3. 8 Quantification of nuclear and cytoplasmic ABCG2 staining in the Bath prostate tissue samples. Immunohistochemical staining of ABCG2 was quantified in the Bath cohort using the proportion and intensity 1 scores for nuclear and cytoplasmic IHC staining. (A) Nuclear ABCG2 staining showed no significant difference between normal and malignant prostate tissues (p= 0.7873). (B) Cytoplasmic ABCG2 staining was significantly reduced in PCa compared to NP tissues (p= 0.048). (C) Nuclear ABCG2 staining was positively associated with increasing primary Gleason grade (p=0.0221). (D) Cytoplasmic ABCG2 staining was

negatively associated with increasing primary Gleason grade, but the results were not significant (p=0.1199). (E) Nuclear ABCG2 staining was positively associated with increasing clinical stage T (p=0.0337). (F) There was no association between cytoplasmic ABCG2 and clinical stage T (p=0.327). (G) There was no association between nuclear ABCG2 and risk of recurrence (p=0.8106). (H) Cytoplasmic ABCG2 staining was negatively associated with a risk of recurrence (p=0.0141). The mean of five random fields was taken per prostate sample. Statistical significance was determined with unpaired T-test for each set of conditions. NP (n=4), PCa (n=26), grade 3(n=9), grade 4(n= 14), T1-2 (n=13), T3-4 (n= 8), recurrent PCa (n=16), non- recurrent PCa (n=8).

Table 3. 5 Nuclear and cytoplasmic ABCG2 staining results with clinical data.

Comparison	**Nuclear ABCG2 staining**		**Cytoplasmic ABCG2 staining**	
	Results	**p. value**	**Results**	**p. value**
Normal vs malignant	No statistically significant difference	0.7873	Lower in malignant	0.048
Primary Gleason grades (3 & 4)	Higher in high grade	0.0221	No statistically significant difference	0.1199
Clinical Stage (T)	Higher in T3-4	0.0337	No statistically significant difference	0.327
5 years Biochemical recurrence	No statistically significant difference	0.8106	Lower in recurrence	0.0141

In summary, nuclear ABCG2 staining was not associated significantly with PCa, but was positively associated with increasing grade and advanced stage. In contrast, cytoplasmic ABCG2 was reduced significantly in PCa and was negatively associated with biochemical recurrence, but not with grade and stage.

3.2.8 ALDH1A1

To test the hypothesis that cytoplasmic ALDH1A1 staining will be increased in PCa compared to NP tissues and will be positively associated with PCa clinical features such as Gleason grade, clinical stage and relapse; IHC was carried out with the Bath prostate tissues.

3.2.8.1 Immunohistochemical staining of ALDH1A1 in normal and malignant Bath prostate samples

Glandular and stromal ALDH1A1 staining was observed in the Bath prostate tissue samples. Nuclear ALDH1A1 staining was shown in the glandular regions of both normal and malignant prostate tissues and ranged from strong (Figure 3.9 B & F, arrows), moderate (Figure 3.9 E, arrow), weak and negative (Figure 3.9 A & C, arrows). Cytoplasmic ALDH1A1 staining was also observed in normal and malignant prostate tissues, but the

intensity of staining was varied, ranging from strong (Figure 3.9 D, arrowhead), moderate (Figure 3.9 E, arrowhead), weak (Figure 3.9 B, arrowhead) and negative (Figure 3.9 A& C). Interestingly, both nuclear and cytoplasmic ALDH1A1 staining was found to be restricted to basal cells of NP tissues (Figure 3.9 B). As was expected in PCa, nuclear and cytoplasmic ALDH1A1 staining, however, was found to be in all prostate gland epithelial cells, including residual basal (Figure 3.9 F, arrow) and luminal cells (Figure 3.9 D&E).

In addition to the glandular ALDH1A1 staining, stromal ALDH1A1 staining was also observed in the Bath prostate tissue samples, with different staining levels, ranging from strong (Figure 3.9 A&C, arrowhead) to weak (Figure 3.9 E). ALDH1A1 is expressed in testis tissues (Deng *et al.*, 2010) and so this study used testis tissues as a positive control for ALDH1A1 and IHC staining showed nuclear and cytoplasmic ALDH1A1 staining in testis tissues (Figure 3.9 G). The negative control showed no significant background staining on prostate samples (Figure 3.9 H, arrow).

3.2.8.2 Association between ALDH1A1 immunostaining with histopathological parameters of prostate cancer in the Bath cohort

Having carried out IHC staining, nuclear and cytoplasmic glandular ALDH1A1 was then quantified using the proportion and intensity 3 score, whereas stromal proportion and intensity ALDH1A1 staining were quantified using proportion 1 and intensity 1 respectively. The range of glandular (nuclear and cytoplasmic) and stromal (proportion and intensity) ALDH1A1 staining varied between 0-2.8, 0-3.8, 0-3, and 0-2.8 respectively. The potential association between ALDH1A1 IHC and histopathological parameters of PCa was then analysed and is described below.

The statistical analysis showed that there was no significant association between nuclear and cytoplasmic ALDH1A1 staining when comparing normal vs malignant prostate tissues, different primary Gleason grades, relapse and clinical stages except T (Table 3.6). ALDH1A1 stromal staining was also not associated significantly in normal vs. malignant prostate tissue, or other PCa histopathological features (Table 3.7). In contrast, there was a negative association between stromal ALDH1A1 (proportion and intensity) staining and biochemical recurrence (Figure 3.10 & Table 3.7).

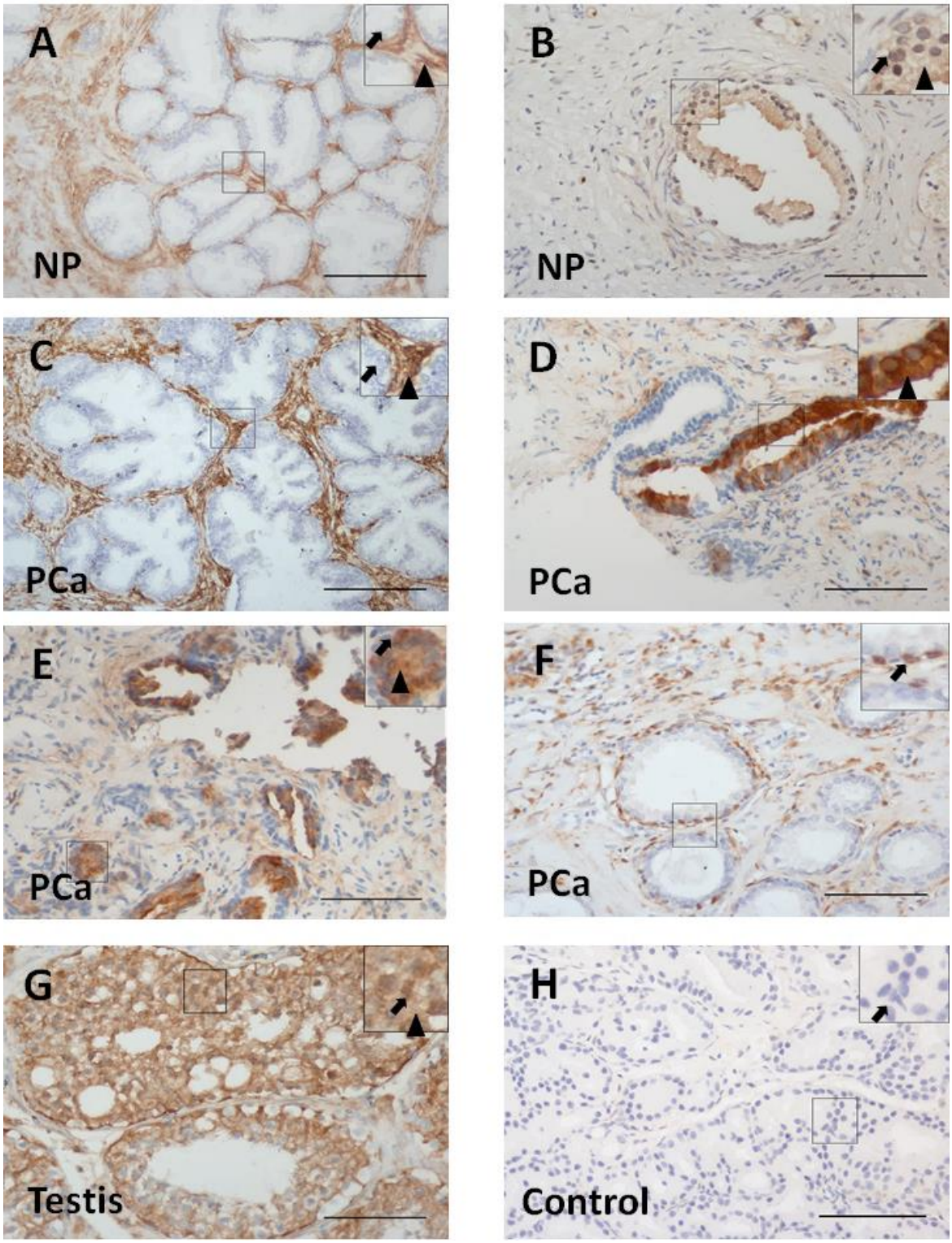

Figure 3. 9 ALDH1A1 was stained heterogeneously in both normal and malignant tissues of the prostate. (A) ALDH1A1 stromal staining (Black arrowhead) and negative glandular region (Black arrow) in NP. (B) Strong nuclear AlDH1A1 staining (Black arrow) and weak cytoplasmic (Black arrowhead) in the basal cell of NP. (C) Strong Stromal (Black arrowhead) with negative glandular (Black arrow) ALDH1A1 staining in PCa. (D) Strong cytoplasmic ALDHIA1 staining (Black arrowhead) in the glandular region of PCa. (E) Moderate nuclear (Black arrow) with cytoplasmic (Black arrowhead) ALDH1A1 staining in the glandular region of PCa. (F) Strong nuclear ALDH1A1 staining (Black arrow) in basal cells of PCa. (G) Strong nuclear (Black arrow) and cytoplasmic (Black arrowhead) ALDH1A1 staining in normal testis tissue. (H) The negative control (no primary antibody) showed no staining (Black arrow) in PCa tissue. Scale bars=100µm.

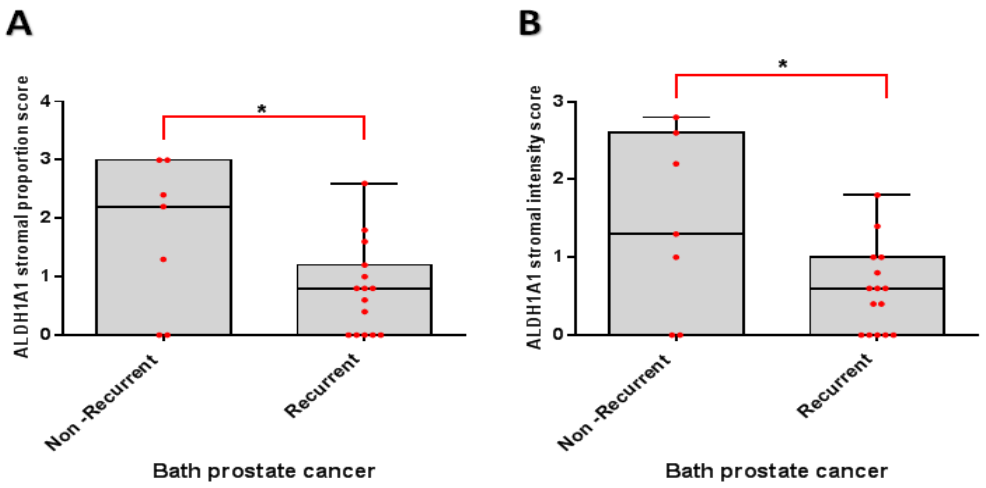

Figure 3. 10 Quantification of stromal ALDH1A1 intensity staining in both normal and malignant Bath prostate tissues. Immunohistochemical staining of ALDH1A1 was quantified in the Bath cohort using intensity 1 scores. (A) ALDH1A1 stromal proportion staining was reduced significantly in PCa recurrent tissues compared to non- recurrent (p=0.0483). (B) ALDH1A1 stromal intensity staining was reduced significantly in PCa recurrent tissues compared to non- recurrent (p= 0.0304). The mean of five random fields was taken per prostate sample. Statistical significance was determined with unpaired T-test for each set of conditions. Recurrent (n=15), non-recurrent (n=7).

Table 3. 6 Glandular nuclear and stromal ALDH1A1 staining results with clinical data.

Comparison	**Nuclear ALDH1A1 staining**		**Cytoplasmic ALDH1A1 staining**	
	Results	**p. value**	**Results**	**p. value**
Normal vs malignant	No statistically significant difference	7.338	No statistically significant difference	0.9081
Primary Gleason grades (3 & 4)	No statistically significant difference	0.3285	No statistically significant difference	0.2896
Clinical Stage (T)	No statistically significant difference	0.0337	No statistically significant difference	0.1629
5 years Biochemical recurrence	No statistically significant difference	0.682	No statistically significant difference	0.6928

Table 3. 7 Stromal proportion and intensity ALDH1A1 staining results with clinical data.

Comparison	ALDH1A1 stromal proportion staining		ALDH1A1 stromal intensity staining	
	Results	**p. value**	**Results**	**p. value**
Normal vs malignant	No statistically significant difference	0.7626	No statistically significant difference	0.8445
Primary Gleason grades (3 & 4)	No statistically significant difference	0.8946	No statistically significant difference	0.9151
Clinical Stage (T)	No statistically significant difference	0.259	No statistically significant difference	0.2349
5 years Biochemical recurrence	Lower in recurrence	0.0483	Lower in recurrence	0.0304

In summary, nuclear and cytoplasmic ALDH1A1 was restricted to the basal layer of NP tissues, whereas, ALDH1A1 was found to be expressed in all prostate gland epithelial cells. Nuclear and cytoplasmic ALDH1A1 staining was not associated with PCa and other histopathological features such as primary Gleason grade, clinical stage and biochemical relapse. Although ALDH1A1 stromal staining was also not associated significantly with PCa, primary Gleason grade and clinical stage, it was negatively associated with biochemical recurrence.

3.2.9 RS1

To test the hypothesis that cytoplasmic RS1 staining will not be altered in PCa compared to NP, but will be positively associated with biochemical relapse, IHC was carried out the TMA prostate tissues.

3.2.9.1 Immunohistochemical staining of RS1 in the normal and malignant Bath prostate samples

In NP tissues, cytoplasmic RS1 staining was found and ranged from strong (Figure 3.11 A, arrowhead) to negative (Figure 3.11 B, arrow). Cytoplasmic RS1 staining was also observed in PCa tissues and the intensity of signal varied widely, from strong (Figure 3.11 D, arrowhead), moderate (Figure 3.11 E, arrowhead), weak (Figure 3.11 F&C, arrowheads) and

negative (Figure 3.11, G arrowhead). In addition to the cytoplasmic staining, the normal and malignant prostate tissue samples all had nuclear RS1 staining, with a variable level of staining between cases, ranging from strong (Figure 3.11 A, C & D, arrows), moderate (Figure 3.11 E, arrow), weak and negative (Figure 3.11 B, F&G, arrows). The negative control (no primary antibody) showed no background staining in PCa tissue (Figure 3.11 H, arrow).

3.2.9.2 Association between RS1 immunostaining and histopathological parameters of prostate cancer in the Bath cohort

The previous PhD student, Ben Sharpe in our laboratory stained 27 PCa and 5 NP tissues and this study added 7 new samples to his data to increase the sample size. Data from both studies are included in the results presented below.

Having carried out IHC staining, the nuclear RS1 staining was quantified using the H-score, whereas, cytoplasmic staining of RS1 was quantified using the cytoplasmic intensity 1. As a secreted protein the nuclear staining might be expected to be background but, as with ABCG2, it was decided to score it as it might provide an internal control that would not be expected to be linked to disease progression. The range of nuclear and cytoplasmic RS1 scores varied between 0- 100 and 0-2.5 respectively. The potential association between RS1 IHC results and histopathological parameters of prostate cancer was then analysed and is described below.

The statistical analysis showed that there was no significant association between nuclear and cytoplasmic RS1 scores when comparing NP tissues to those with malignancies as well as different primary Gleason grades, clinical stages (Table 3.8). Nuclear RS1 staining was not associated with biochemical relapse (Figure 3.12 &Table 3. 8). Cytoplasmic RS1 staining was found to be higher in PCa relapsed patients compared to those with non- relapsed, but the results were not significant (Figure 3.12 &Table 3. 8).

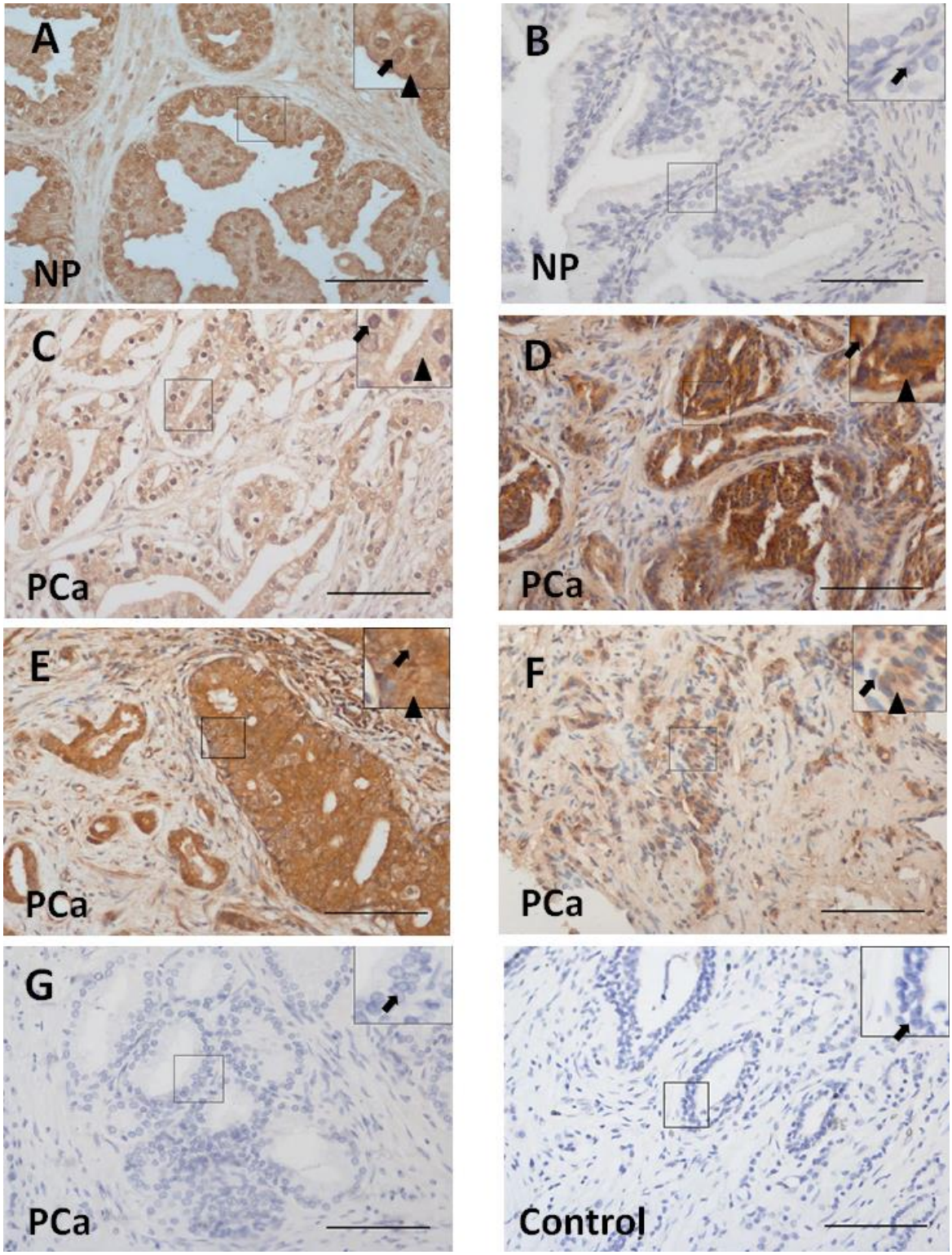

Figure 3. 11 RS1 staining in samples from the Bath cohort. RS1 was stained heterogeneously in both normal and malignant tissues of the prostate. (A) Strong nuclear (Black arrow) and cytoplasmic (Black arrowhead) RS1 staining in NP. (B) Negative RS1 staining in NP. (C) Strong nuclear (Black arrow) and weak cytoplasmic (Black arrowhead) RS1 in PCa. (D) Strong nuclear (Black arrow) and cytoplasmic (Black arrowhead) RS1 staining in PCa. (E) Moderate nuclear (Black arrow) and cytoplasmic (Black arrowhead) RS1 staining in PCa. (F) Weak cytoplasmic (Black arrowhead) and negative nuclear RS1 staining in PCa. (G) Negative nuclear and cytoplasmic RS1 staining in PCa. (H) Negative control (no primary antibody added) showed no background staining (Black arrow) in PCa. Scale bars=100µm.

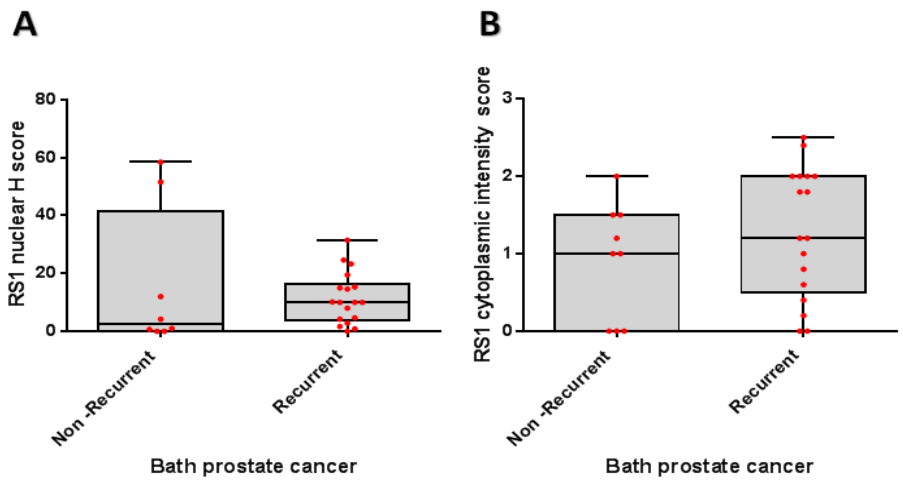

Figure 3. 12 Quantification of nuclear and cytoplasmic RS1 staining in both normal and malignant Bath prostate tissues. Immunohistochemical staining of RS1 was quantified in the Bath cohort using H-score and cytoplasmic intensity the scores respectively. (A) Nuclear RS1 staining was not associated with recurrence (p=0.4916). (B) Cytoplasmic RS1 was higher in recurrent PCa tissue compared to non- recurrent PCa tissues but the result was not significant (P=0.2711). The mean of five random fields was taken per prostate sample. Statistical significance was determined with unpaired T-test for each set of conditions. Recurrent PCa (n=17) and non-recurrent PCa (n=8).

Table 3. 8 Nuclear and cytoplasmic RS1 staining results with clinical data

Comparison	**Nuclear RS1 staining**		**Cytoplasmic RS1 staining**	
	Results	**p. value**	**Results**	**p. value**
Normal vs malignant	No statistically significant difference	0.3058	No statistically significant difference	0.2492
Primary Gleason grades (3 & 4)	No statistically significant difference	0.7297	No statistically significant difference	0.7811
Stage (T)	No statistically significant difference	0.2364	No statistically significant difference	0.8999
5 years Biochemical recurrence	No statistically significant difference	0.4916	No statistically significant difference	0.2711

In summary, nuclear and cytoplasmic RS1 staining was not associated significantly with PCa and other histopathological and clinical features of PCa.

3.4 Discussion

3.4.1 Summary of the results

The expression of five candidate biomarkers, β-catenin, NDRG1, ABCG2, ALDH1A1 and RS1 were investigated in prostate tissues samples from the Bath cohort, using IHC to determine if there was an association between their IHC levels in normal vs. malignant prostate tissues and/or with other histopathological and clinical features of PCa, including primary Gleason grade, clinical stage and biochemical recurrence. The key findings for these potential biomarkers are summarised in Table 3.9. It was decided to discuss the results from each potential biomarker in detail in Chapter 4 after the analysis had been carried out with the larger cohort, rather than discuss the preliminary results here and repeat the discussion in the next Chapter.

Table 3. 9 Summary of key findings for each candidate biomarker in this chapter

Biomarkers	Key Findings
β-catenin	❖ Nuclear B-catenin, but not cytoplasmic, staining was decreased significantly in PCa. ❖ Nuclear and cytoplasmic staining was negatively associated with increasing primary Gleason grade, but not stage or relapse.
NDRG1	❖ Decreased membranous and increased nucleocytoplasmic localisation of NDRG1 was significantly associated with PCa, but not with grade, stage and relapse. ❖ Changes in nuclear or cytoplasmic NDRG1 levels were not significantly associated with clinical features of PCa.
ABCG2	❖ Cytoplasmic ABCG2 staining was reduced significantly in PCa and was negatively associated with biochemical relapse. ❖ Nuclear ABCG2 staining was positively associated with increasing primary Gleason grade and stage.
ALDH1A1	❖ Changes in cytoplasmic and nuclear ALDH1A1 staining in the glands was not significantly associated with clinical features of PCa. ❖ ALDH1A1 stromal proportion and intensity staining were negatively associated with biochemical relapse, but not normal vs malignant, grade and stage.
RS1	❖ Changes in Cytoplasmic and nuclear RS1 staining was not significantly associated with clinical features of PCa.

3.4.2 Limitations of using a cohort with a small number of clinical samples

The expression of the five candidate biomarkers has been analysed using samples from the Bath cohort and some interesting changes observed. However, a key limitation of this data is that the Bath cohort consists of a small number of samples, 5 NP and 34 PCa tissues. In addition, NP tissue samples lacked clinical information about age, so they might be from different aged patients than the cancer samples. For this reason, it was decided that the results described above should be treated as preliminary until repeated using a cohort with a much higher sample size. This was carried out in the next chapter by using a prostate TMA with 96 samples (16 NP and 80 PCa).

It was decided to discuss the results from each potential biomarker in detail in chapter 4 after the analysis had been carried out with the larger cohort, rather than discuss the preliminary results here and repeat the discussion in the next chapter.

3.4.3 Conclusion

The preliminary data presented in this chapter suggests that β- catenin and ABCG2 expression are reduced in PCa compared to NP and are negatively associated with increasing Gleason grade. In contrast, changing the localisation of NDRG1 from membranous to nucleocytoplasmic is associated positively with PCa. Three candidate biomarkers showed either a negative, such as ABCG2 and ALDH1A1 (stromal) expression, or positive association, such as cytoplasmic RS1 expression, with relapse. No candidate biomarker was associated significantly with clinical stage. This interesting preliminary data provided support to repeat the staining of these potential biomarkers using a TMA with a much larger sample size in chapter 4.

CHAPTER FOUR

ASSESSMENT OF THE CANDIDATE PROTEINS EXPRESSION IN THE TISSUE MICROARRAY COHORT

4. Assessment of the candidate proteins expression in the tissue microarray cohort

4.1 Introduction

Having completed a first round of analysis of expression of the candidate biomarkers using tissue from the Bath cohort, the next step was to repeat the analysis using a larger cohort of samples. One way of efficiently staining large cohorts of tissue is to use a TMA slide.

A TMA is a high-throughput technique that can be used in histopathological fields to evaluate molecular markers in a number of diseases, including cancer, using common laboratory methods such as IHC, ISH and fluorescent *in situ* hybridization (FISH) (Voduc *et al.*, 2008). This technique was first described in 1988 by Kononen (Kononen *et al.*, 1998) and there are many scientific and economic benefits, as well as some drawbacks for using the TMA technique. First, TMA slides have normally many tissue samples coming from different donor samples that were re-embedded in a single microarray block (Jawhar, 2009), this is then sectioned, allowing many samples to be analysed on a single slide. Second, the use of a tiny core per case reduces the amount of tissue that comes from each block, allowing the samples to be used in other TMA slides (Voduc *et al.*, 2008). In addition, all TMA tissues samples are on a single slide so are exposed to the same experimental conditions, including antigen retrieval, temperature, incubation time, washing and reagent concentration (Jawhar, 2009; Shergill *et al.*, 2004) and that may reduce technical errors. Finally, the use of this technique may have some economic benefits, including low cost and a reduced amount of researcher time (Jawhar, 2009; Shergill *et al.*, 2004).

There are some disadvantages have been noticed from using TMAs in clinical research. The use of a tiny core per case may not give a representative description of the whole tissue samples (Shergill *et al.*, 2004), especially in heterogeneous diseases such as PCa and for that reason a previous comparison between using TMA and a whole section of prostate suggested that the optimal number of tissue cores in the TMA which may predict the outcome of the whole tissues of PCa is about 3-4 cores (Rubin *et al.*, 2002). However, another PCa study with three different sets of TMAs, (1637 PCa cores each), has reported the use of a single 0.6 mm core per case sufficient for biomarker evaluation, whereas, using multiple cores per case may give a bias toward marker positivity (Tennstedt *et al.*, 2012). In addition, the tiny cores may be lost due to exposure to pretreatment, including antigen retrieval and washing

steps. Finally, the slides can lack clinical information which may help to understand the outcome of diseases, such as clinical data regarding the relapse and non-relapse status of the sample. In general, the advantages of using a TMA appeared to outweigh the disadvantages so it was decided to use one for the next stage of this project.

This study used a commercially available TMA with a large number of prostate tissue samples. It contained NP and different grades and stages of PCa. This made it possible to test the expression of the candidate biomarkers in a large number of samples and repeat the results using a completely independent set of patient samples. One disadvantage of this TMA is that it lacks clinical data regarding PCa relapse and non-relapse. This TMA was used to examine the potential biomarkers, that have been selected and tested previously in Chapter 3, and then to analyse the results and test for association with the clinical data and finally test the hypothesis proposed in Chapter 3.

4.1.1 Aims

1. To detect the expression levels of the potential biomarkers described previously in chapter 3 in a large TMA prostate cohort.
2. To establish if the expression of these potential biomarkers correlates with clinical features of PCa, including primary Gleason grade and clinical stage.
3. Compare to the results from the Bath cohort and test the hypothesis, proposed in Chapter 3, regarding the expression of the potential biomarkers in PCa.

4.2 Results

In this chapter, the expression of five candidate biomarkers β-catenin, NDRG1, ABCG2, ALDH1A1, and RS1 was examined using IHC on the TMA prostate tissues. The structure of the TMA cohort and the results for each candidate biomarker are described below. The data obtained from the TMA cohort was then compared to that from the Bath cohort and the hypothesis, proposed in Chapter 3 that describe their predicted expression in PCa, tested.

4.2.1 Structure of the TMA cohort

The TMA slides were obtained from US Biomax and consisted of 96 prostate cases, 80 of them were PCa, whereas the rest were normal tissue or normal tissue that was adjacent to PCa, termed adjacent normal (8 cases for each). Each prostate case was represented with two core tissue biopsies to form a total of 192 cores. The clinical data of the patients are shown in Table 4.1.

Table 4. 1 Clinical data of the TMA prostate sample cohort.

The TMA Clinical data		Number	%
Number of samples	Normal	16	
	Malignant	80	
Age range	Normal	21-68	
	Malignant	20-85	
Primary Gleason grade	3	13	16.25
	4	46	57.5
	5	18	23. 75
	ND	3	2.5
Clinical stage	I	6	7.5
	II	37	46.3
	III	14	17.5
	IV	22	27.5
	ND	1	1.3
T category	T1-2	51	63.8
	T3-4	28	35
	ND	1	1.2
N category	N0	65	81.2
	N1	14	17.5
	ND	1	1.3
M category	M0	64	80
	M1	15	18.7
	ND	1	1.3

4.2.2 β-catenin

β-catenin IHC was carried out with the TMA prostate tissue samples and, as with the Bath cohort, the analysis focused on nuclear and cytoplasmic β-catenin IHC staining.

4.2.2.1 Immunohistochemical staining of β-catenin in the normal and malignant TMA prostate samples

IHC staining showed membranous, cytoplasmic and nuclear β-catenin staining in the TMA prostate tissues (Figure 4.1). Nuclear β-catenin staining was found in NP tissues with a variable level of staining, ranging from strong (Figure 4.1 A, arrow), moderate (Figure 4.1 B, arrow), and weak in some cases (Figure 4.1 C, arrow). Nuclear β-catenin staining was also observed in PCa tissues and the intensity of signal varied widely, from strong and

widespread (Figure 4.1 D, arrow) to weak and scattered (Figure 4.1 F, arrow) or negative in some cases (Figure 4.1 G, arrow).

In addition to the nuclear staining, the normal and malignant prostate tissue samples had cytoplasmic β-catenin staining, with variable staining levels between cases, ranging from strong (Figure 4.1. D & E, arrowheads), moderate (Figure 4.1 A, arrowhead) and weak (Figure 4.1 B, C, F &G, arrowheads). A negative control (no primary antibody) showed no significant background staining in prostate tissue (Figure 4.1 H, arrow).

4.2.2.2 Association between β-catenin immunostaining and histopathological parameters of prostate cancer in the TMA cohort

Having carried out IHC staining, the nuclear and cytoplasmic β-catenin staining was then quantified using the H & proportion and intensity 1 scores, respectively, as carried out for the Bath cohort samples and then compared between normal vs. malignant prostate tissues, primary Gleason grades, clinical stages. The range of nuclear β-catenin and cytoplasmic scores varied between 0 - 165 and 2 - 7, respectively (Figure 4.2). The potential association between β-catenin IHC results and histopathological parameters of PCa was then analysed and is described below.

The first analysis looked at the β-catenin staining in normal vs. malignant prostate tissues. Quantification of the IHC staining showed a significant reduction of nuclear and cytoplasmic β-catenin staining in malignant prostate tissues compared to those with a normal nature ($p < 0.0001$& 0.0412, respectively) (Figure 4.2 A&B & Table 4.2). Decreased nuclear and cytoplasmic β-catenin was also associated with increasing primary Gleason grade ($p= 0.0004$, 0.0002 respectively) (Figure 4.2 C&D & Table 4.2). Analysis of data, using the multi-comparison Tukey's tests showed reduced nuclear β-catenin staining significantly when comparing PCa tissues with a primary Gleason grade 3 to those with a grade 4 ($p=0.0254$) or a grade 5 ($p=0.0003$). Nuclear β-catenin staining also showed a significant difference between primary Gleason grade 5 & 4 ($p= 0.0494$). Cytoplasmic β-catenin staining was decreased significantly in PCa tissues with a primary Gleason grade 5 compared to those with a grade 3 (0.0004) or a grade 4 ($p= 0.0030$), but not between primary Gleason grade 3 and 4 ($p=0.2284$) (Figure 4.2 C&D & Table 4.2). There was no significant difference between β-catenin staining (nuclear and cytoplasmic) and clinical stage (TNM) (Table 4.2), except nuclear β-catenin staining showed a negative association with PCa metastasis ($P= 0.0014$) (Figure 4.2 F & Table 4.2).

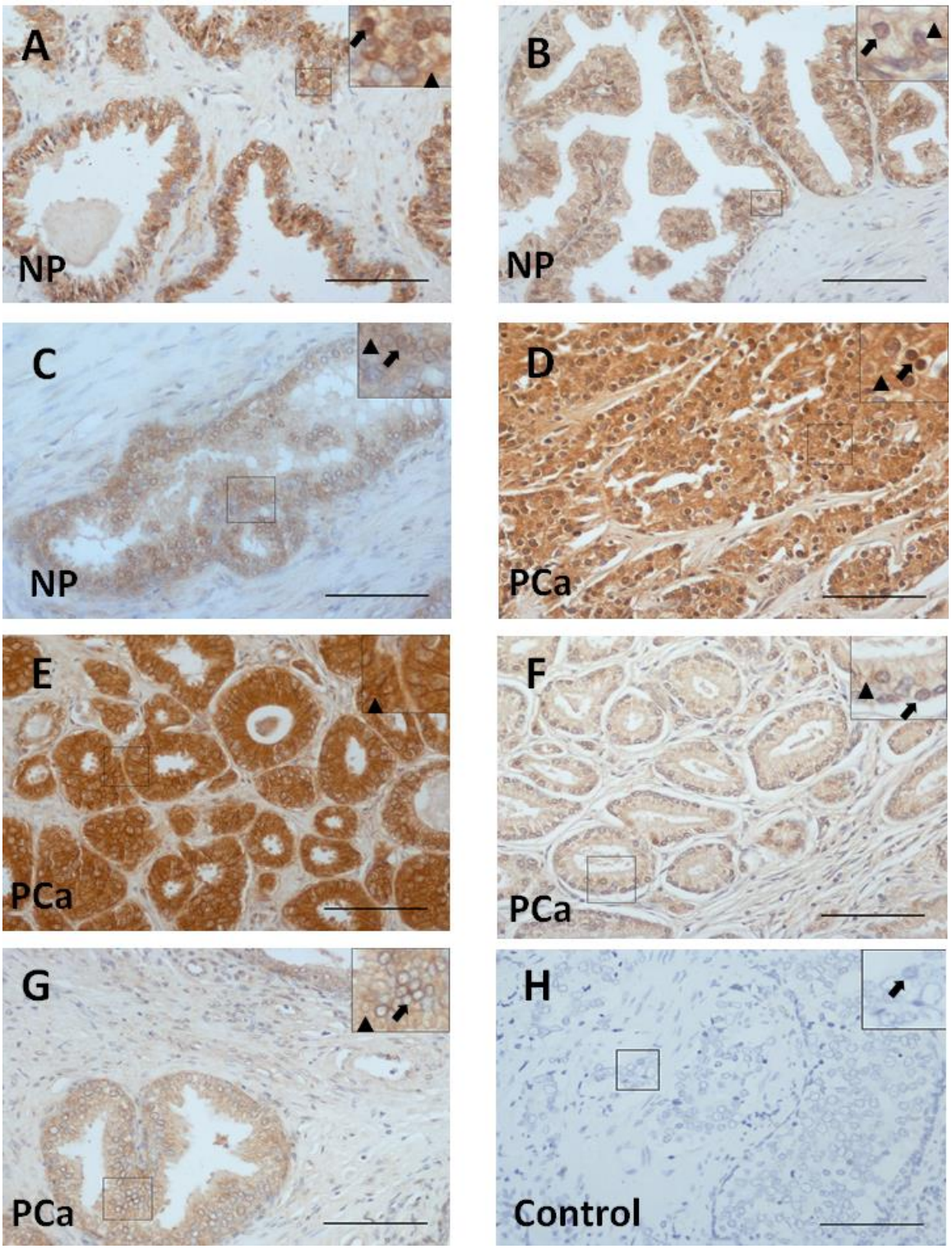

Figure 4. 1 β-catenin staining in samples from the TMA cohort. β-catenin was stained heterogeneously in normal and malignant tissues of the prostate. (A) Strong nuclear (Black arrow) and moderate cytoplasmic (Black arrowhead) β-catenin staining in NP. (B) Moderate nuclear (Black arrow) and weak cytoplasmic (Black arrowhead) β-catenin staining in NP. (C) Weak nuclear (Black arrow) and cytoplasmic (Black arrowhead) β-catenin staining in NP. (D) Strong nuclear (Black arrow) and cytoplasmic (Black arrowhead) β-catenin staining in PCa. (E) Strong cytoplasmic β-catenin staining (Black arrowhead) in PCa. (F) Weak nuclear (Black arrow) and cytoplasmic (Black arrowhead) β-catenin staining in PCa. (G) Weak cytoplasmic (Black arrowhead) with some negative nuclear (Black arrow) β-catenin staining in PCa. (H) Negative control (no primary antibody added) showed no background staining (Black arrow) in PCa. Scale bars=100µm.

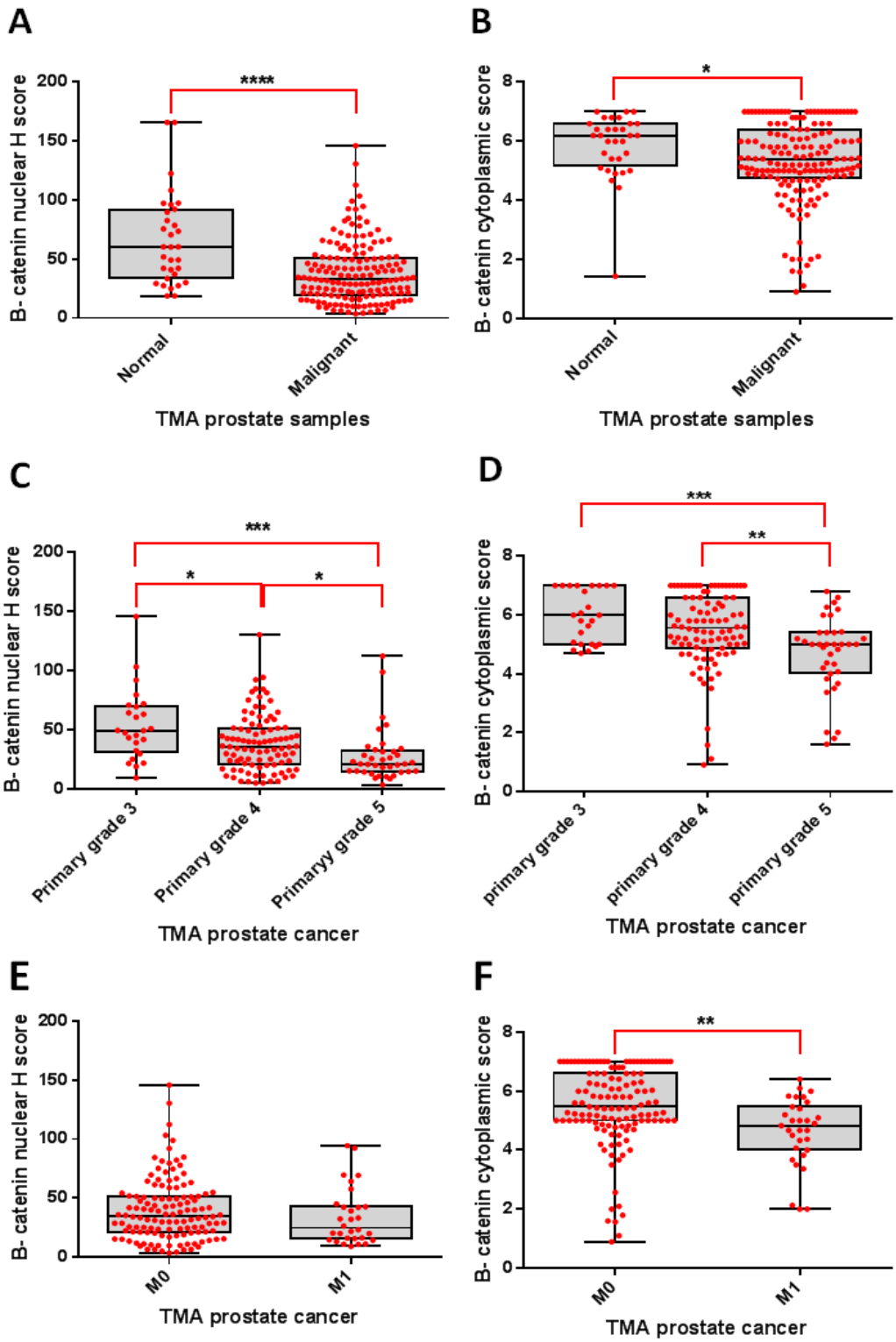

Figure 4. 2 Quantification of nuclear and cytoplasmic β-catenin staining in both normal and malignant TMA prostate tissues. Immunohistochemical β-catenin staining was quantified in the TMA cohort using H and the proportion and intensity 1 scores for nuclear and cytoplasmic IHC staining respectively. (A) Nuclear β-catenin staining was significantly reduced in PCa compared with NP tissues (p= < 0.0001). (B) A significant reduction of cytoplasmic β-catenin staining was in the TMA malignant prostate tissues compared with NP (p= 0.0412). (C) Nuclear β-catenin staining showed a significant difference among primary Gleason grades (p= 0.0004). Multi comparison Tukey's tests showed a significant reduction in nuclear β-catenin staining when comparing grade 5 tissues to those with a grade 3 (p=0.0004) or grade 4 (p=0.0492). This reduction was significant between grade 4 and 3 (p=0.0254). (D) Cytoplasmic β-catenin staining showed a significant difference among Gleason grade groups (p= 0.0002). A multi comparison of Tukey's tests showed that cytoplasmic β-catenin

staining was significantly reduced in grade 5 compared to grade 3 (p= 0.0004) or grade 4 (p=0.0030). There was no association between cytoplasmic β-catenin staining when comparing Gleason grades 4 and 3 (p= 0.2284). (E) Nuclear β-catenin staining was not associated with clinical stage M (P=0.2795). (F) Cytoplasmic β-catenin staining was negatively associated with metastasis (p=0.0014). Data represent the mean of five random images per case. Unpaired or one-way ANOVA tests were conducted to determine the statistical difference for each set of conditions. NP (n=16), PCa (n=80), grade 3 (n=13), grade 4 (n=46), grade 5 (n=18), M0 (n= 64) and M1 (n=15).

Table 4. 2 Nuclear and cytoplasmic β-catenin staining results with clinical data.

Comparison	**Nuclear β-catenin staining**			**Cytoplasmic β-catenin staining**		
	Results		**p. value**	**Results**		**p. value**
Normal vs malignant	Lower in malignant		< 0.0001	Lower in malignant		0.0412
Primary Gleason grades (3, 4 &5)	Lower in high Gleason grade	Anova test	0.0004	Lower in high Gleason grade	Anova test	0.0002
		Grade 4 vs. Grade 3	0.0254		Grade 4 vs. Grade 3	0.2284
		Grade 5 vs. Grade 3	0.0003		Grade 5 vs. Grade 3	0.0004
		Grade 5 vs. Grade 4	0.0492		Grade 5 vs. Grade 4	0.0030
Stage (T)	No statistically significant difference		0.9434	No statistically significant difference		0.4809
Stage (M)	No statistically significant difference		0.275	No statistically significant difference		0.084
Stage (N)	No statistically significant difference		0.2795	Lower in PCa with metastasis		0.0014

In summary, nuclear and cytoplasmic β-catenin staining of the TMA samples was reduced significantly in PCa and was negatively associated with increasing primary Gleason grade. Cytoplasmic β-catenin staining was also negatively associated with metastasis.

4.2.2.3 β-catenin immunostaining in the Bath and TMA cohorts: testing the hypothesis

The results from the two cohorts and if the hypothesis is accepted or rejected is summarised in Table 4.3.

Table 4. 3 The summary of β-catenin results in the Bath and TMA cohorts

Parameters	**Localisation**	**Hypothesis**	**Bath**	**TMA**	**Results Confirmed?**	**Hypothesis Accepted**
Normal vs. Cancer	Nuclear	Lower in PCa	Lower in PCa	Lower in PCa	Yes	Yes
	Cytoplasmic	N/A	Trend to be lower in PCa	Lower in PCa	Yes	N/A

Primary Gleason grades	Nuclear	Lower in high grade	Lower in high grade	Lower in high grade	Yes	Yes
	Cytoplasmic	N/A	Lower in high grade	Lower in high grade	Yes	N/A
Stage T	Nuclear	Lower inT3-4	No significant difference	No significant difference	Yes	No
	Cytoplasmic	N/A	No significant difference	No significant difference	Yes	N/A
Stage M	Nuclear	Lower in M1	Not tested	No significant difference	N/A	NO
	Cytoplasmic	N/A	Not tested	Lower in M1	N/A	N/A
Stage N	Nuclear	Lower in N1	Not tested	No significant difference	N/A	NO
	Cytoplasmic	N/A	Not tested	No significant difference	N/A	N/A
Relapsed vs. Non-relapsed	Nuclear	Lower in relapse	No significant difference	Not tested	N/A	NO
	Cytoplasmic	N/A	No significant difference	Not tested	N/A	NO

4.2.3 NDRG1

NDRG1 IHC was carried out with the TMA prostate tissue samples and, as with the Bath cohort, the analysis focused on NDRG1 localisation and staining using IHC.

4.2.3.1 Immunohistochemical localisation and expression of NDRG1 in normal and malignant TMA prostate samples

The IHC result showed membranous NDRG1 staining in the TMA prostate tissue samples with variable levels of staining, ranging from strong (4.3 A, red arrowhead), moderate (Figure 4.3 B& C, red arrowheads), weak (Figure 4.3 F, red arrowhead) and negative (Figure 4.3, G). Cytoplasmic NDRG1 staining was also observed in normal and malignant prostate tissues and the intensity of signal varied widely, ranging from strong (Figure 4.3 E, black arrowhead), moderate (Figure 4.3 C, D & F, black arrowheads), weak (Figure 4.3 A, B, black arrowheads) and negative (Figure 4.3 G). In addition to membranous and cytoplasmic staining, normal and malignant prostate tissue had nuclear NDRG1 staining with variable levels of staining, ranging from strong (Figure 4.3 D, arrow), moderate (Figure 4.3 B, arrow), weak (Figure 4.3 F, arrow) and negative (Figure 4.3, G). A negative control (no primary antibody) showed no significant background staining in prostate tissue (Figure 4.3 H, arrow).

4.2.3.2 Association between NDRG1 immunostaining and histopathological parameters of prostate cancer in the TMA cohort

Having carried out IHC staining, the membranous, cytoplasmic and nuclear localisation of NDRG1 staining was then quantified and compared to the histopathological and clinical data available for the TMA cohort. Having scored the localisation, the amount of staining was then quantified and the potential association between NDRG1 results and histopathological parameters of PCa analysed.

4.2.3.2.1 NDRG1 localisation

Three different NDRG1localisations were observed in the TMA prostate tissues using IHC, including predominant membranous, predominant nucleocytoplasmic and negative. The statistical analysis looked first at the NDRG1 localisation in normal vs. malignant prostate tissues. Quantification of the IHC staining showed a significant difference between NDRG1 localisation in normal vs malignant prostate tissues ($p = <0.0001$). Decreased membranous and increased nucleocytoplasmic NDRG1 staining was observed in PCa tissue compared to those with a normal nature (Figure 4.4 A). Membranous NDRG1 staining was predominantly localised in 69% of PCa compared to 35% of NP tissues (Figure 4.4 A). In contrast, nucleocytoplasmic NDRG1 localisation was found in 61% of PCa compared to 22% of NP.

In addition, NDRGI localisation showed significantly associated with primary Gleason grade ($p=0.0021$). The frequency distribution tests showed membranous NDRG1 localisation negatively associated with increasing primary Gleason grade, whereas, nucleocytoplasmic localisation was positively associated with primary Gleason grade (Figure 4.4 B).

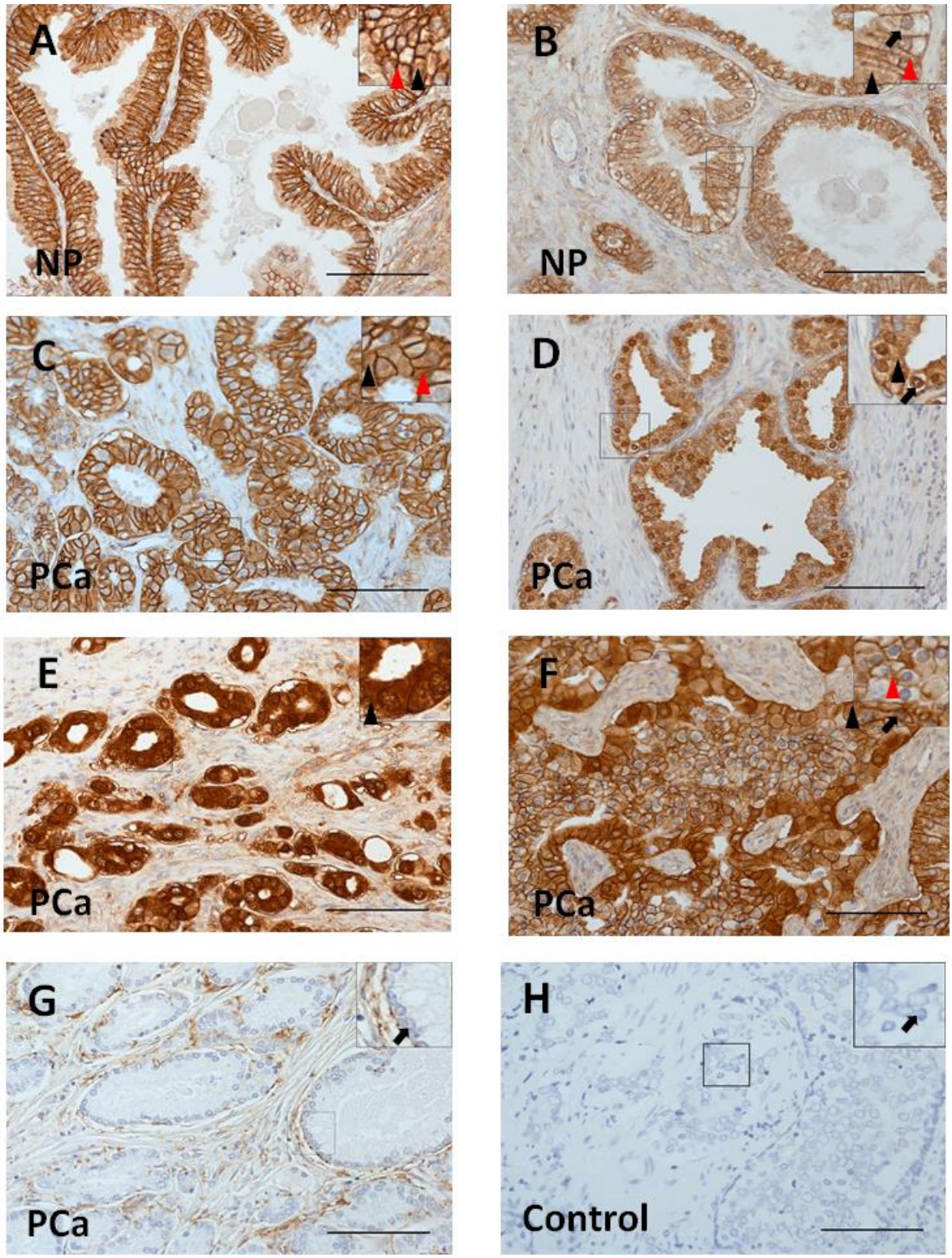

Figure 4. 3 NDRG1 staining in samples from the TMA cohort. NDRG1 staining was found heterogeneously in both normal and malignant tissues of the prostate. (A) Predominant membranous (Red arrowhead) and cytoplasmic (Black arrowhead) NDRG1 staining in NP. (B) Moderate nuclear (Black arrow), membranous (Red arrowhead) and weak cytoplasmic (Black arrowhead) NDRG1 staining in NP. (C) Moderate membranous (red arrowhead) and cytoplasmic (Black arrowhead) NDRG1 staining in PCa. (D)Strong nuclear (Black arrow) with moderate cytoplasmic (Black arrowhead) NDRG1 staining in PCa. (E) Strong cytoplasmic (Black arrowhead) NDRG1 staining in PCa. (F) Weak nuclear (Black arrow), membranous (Red arrowhead) and moderate cytoplasmic (Black arrowhead) NDRG1 staining in PCa. (G) negative staining for NDRG1 (Black

arrow) in PCa. (H) The negative control (no primary antibody) showed no staining (Black arrow) in prostate tissue. Scale bars=100µm.

The membranous NDRG1 localisation was found in 80% of primary Gleason grade 3 and a quarter of Gleason grade 5 tissues. In contrast, three-quarters of tissues with a Gleason grade 5 had nucleocytoplasmic NDRG1 localisation, whereas, NDRG1 was found to be predominantly nucleocytoplasmic localised in 20% of PCa with a Gleason grade 3 (Figure 4.4 B). In contrast, there was no significant difference between NDRG1 localisation and clinical stage (TNM) (Figure 4.4 C, D&E).

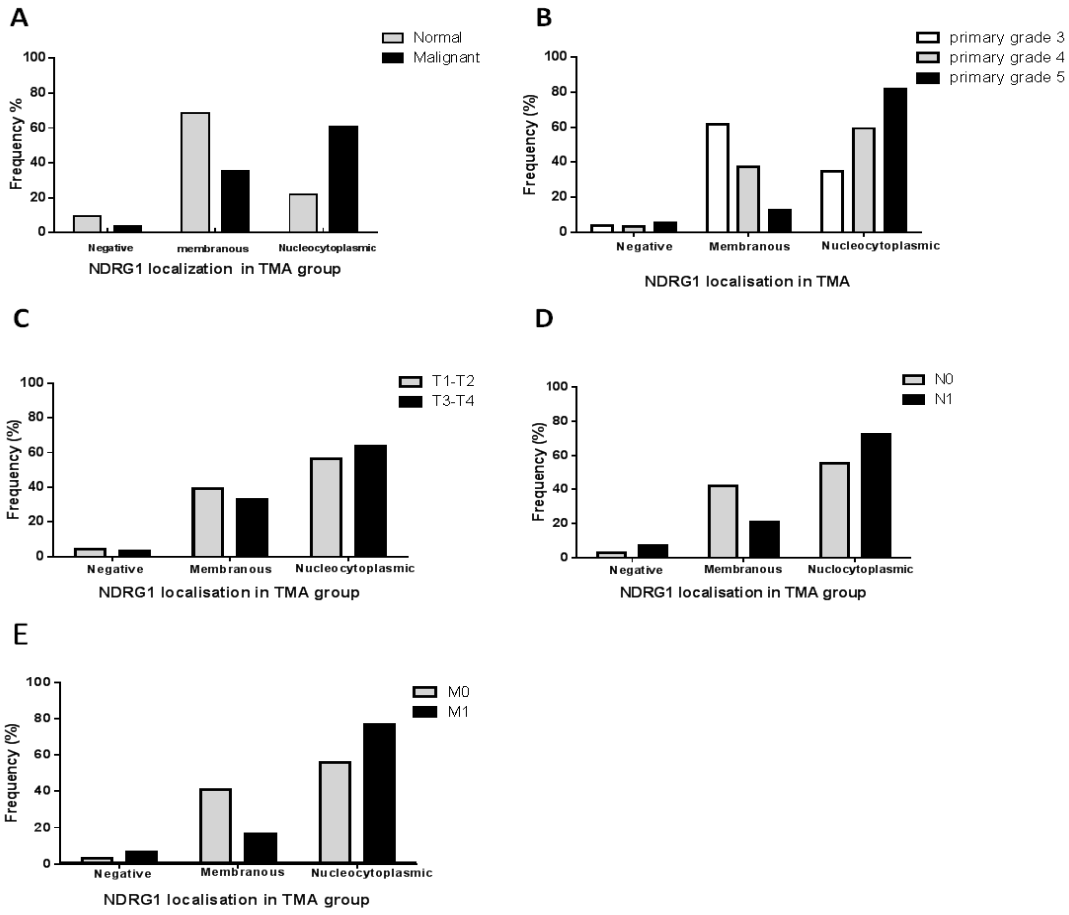

Figure 4.4 Localisation of NDRG1 in TMA prostate samples. Immunohistochemical staining of NDRG1 localisation was quantified in the TMA group using the localisation 1 score. (A) Predominant membranous NDRG1 staining was higher in NP compared to PCa, whereas, predominant nucleocytoplasmic NDRG1 staining was higher in PCa compared to NP tissues (P= <0.0001). (B) Reduced membranous and increased nucleocytoplasmic NDRG1 localisation was positively associated with increasing primary Gleason grade (P=0.0021). (C) There was no clear difference between NDRG1 localisation and stage T (P=0.3770). (D) There was no clear difference between NDRG1 localisation and stage N (P=8161). (E) There was no clear difference between NDRG1 localisation and stage M (P=0.1392). The mean of five random fields was taken per prostate sample. Statistical significance was determined with Chi-square and frequency tests for each set of conditions. NP (n=16), PCa (n=80), grade 3 (n=13), grade 4 (n=46), grade 5 (n=18), M0 (n= 64) and M1 (n=15).

4.2.3.2.2 NDRG1 staining

Having quantified the localisation, the amount of nuclear and cytoplasmic NDRG1 staining was quantified, as carried out for the Bath cohort, and then compared between normal vs. malignant prostate, different primary Gleason grades, clinical stages, using the clinical data available for the TMA cohort. The range of nuclear and cytoplasmic NDRG1 scores varied between 0-7 (Figure 4.5).

The first analysis looked at the staining of NDRG1 in the TMA prostate tissues. Quantification of the IHC showed nuclear NDRG1 staining increased significantly in PCa compared to NP tissues (p=0.0002) (Figure 4.5. A & Table 4.4). In contrast, a significant reduction for cytoplasmic NDRG1 staining was observed in PCa compared to NP tissues (p=<0.0001) (Figure 4.5 B & Table 4.4). There was a significant difference in nuclear NDRG1 scores among different primary Gleason grades (p= 0.0226) (Figure 4.5 C & Table 4.4). Analysis of the IHC, using Tukey's multiple comparisons tests showed nuclear NDRG1 staining significantly decreased in PCa tissues with a primary Gleason grade 3 compared to those with a grade 4 (p= 0.0395) or a grade 5 (p= 0.0271) (Figure 4.5 C & Table 4.4), but not between a Gleason grade 4 and 5 (p= 0.8083) (Figure 4.5 C & Table 4.4). Cytoplasmic NDRG1 staining was observed to a trend toward lower in primary Gleason Grade 5, but the results were not significant (p= 0.1742) (Figure 4.5 C & Table 4.4).

In addition, nuclear NDRG1 staining was increased significantly in advanced PCa stage compared to localised PCa (T3-4 vs T1-2) and N (N1 vs N0) (p= 0.0214 & 0.0039 respectively) (Figure 4.6 A &B & Table 4.4). However, there was no significant association between nuclear NDRG1 staining and PCa metastasis (p= 0.1627) (Figure 4.6 C & Table 4.4). Cytoplasmic NDRG1 staining was not associated with clinical stage TNM (Table 4.4).

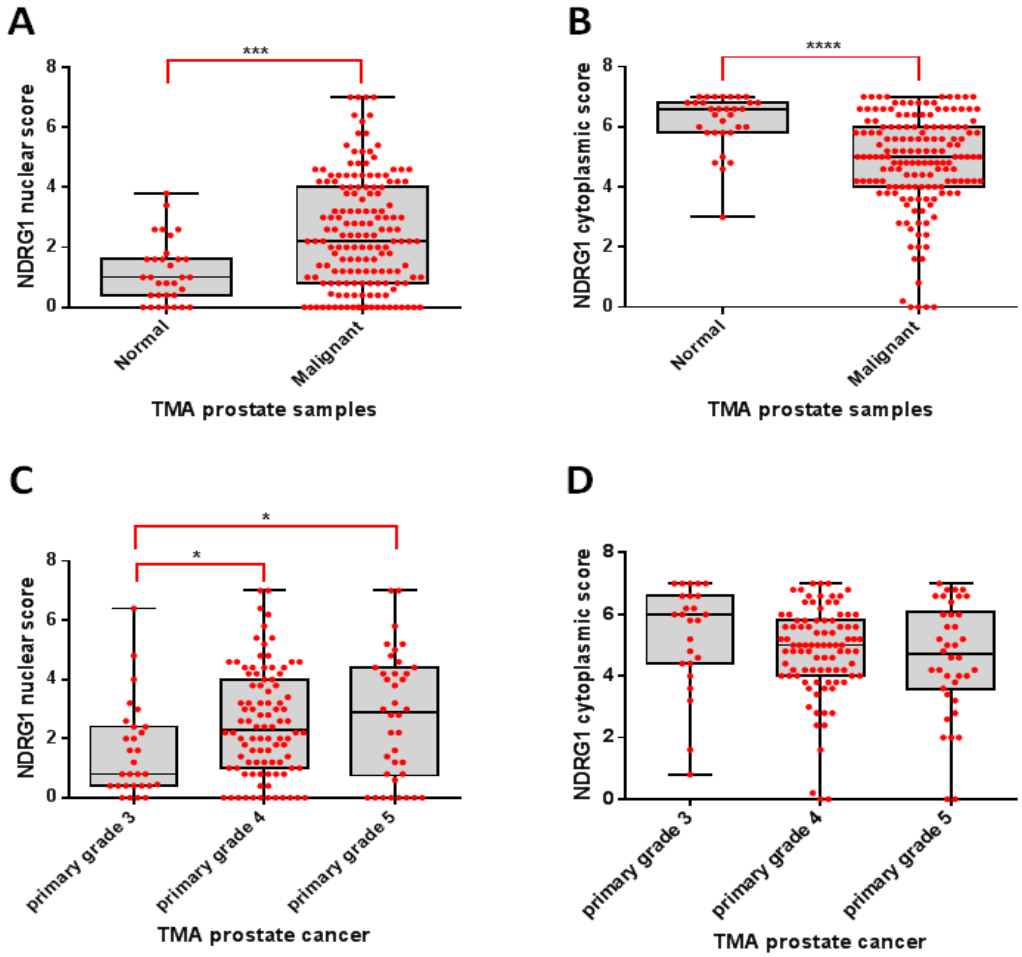

Figure 4. 5 Quantification of nuclear and cytoplasmic NDRG1 staining in both normal and malignant TMA prostate tissues. IHC staining of NDRG1 was quantified in the TMA cohort using the proportion and intensity 1 score for nuclear and cytoplasmic IHC staining. (A) Nuclear NDRG1 staining was significantly increased in PCa compared to NP (p= 0.0002). (B) Cytoplasmic NDRG1 staining was significantly decreased in PCa compared to NP (P= < 0.0001). (C) NDRG1 nuclear staining showed a significant difference among primary Gleason grades (p= 0.0226) and the multicomparisonTukey's tests showed nuclear NDRG1 staining lower in primary Gleason grade 3 compared to grade 4 (p= 0.0395) or grade 5 (p= 0.0271). (B) Cytoplasmic NDRG1 staining was not associated significantly with primary Gleason score groups (p= 0.01742). Unpaired or one-way ANOVA tests were conducted to determine the statistical difference for each set of conditions. NP (n=16), PCa (n=80), grade 3 (n=13), grade 4 (n=46), grade 5 (n=18).

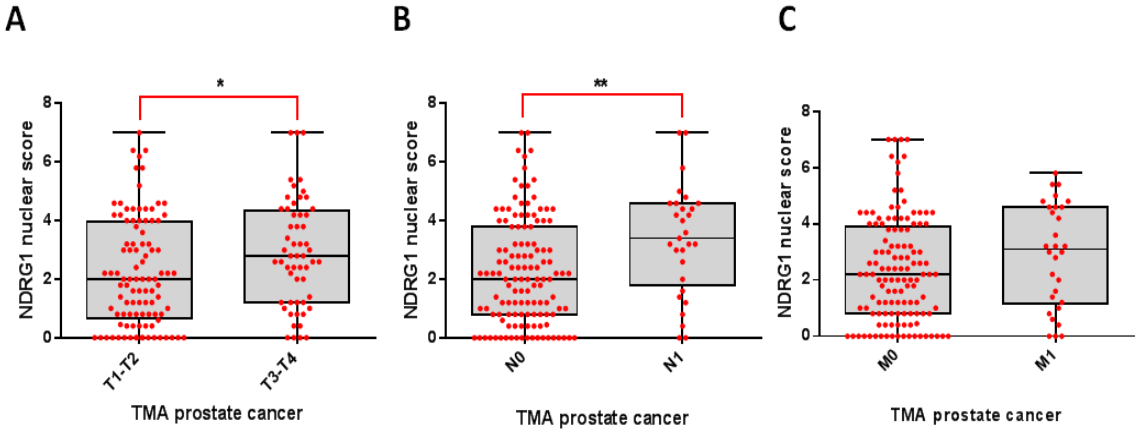

Figure 4. 6 Quantification of nuclear NDRG1 staining in PCa stages. Nuclear NDRG1 staining was quantified in the TMA group using the proportion and intensity 1 score. (A) Nuclear NDRG1 staining was significantly increased in the advanced stages (T3-4) compared to localised PCa (T1-2) (p= 0.0214). (B) Nuclear NDRG1

staining was significantly associated with lymph node status (p= 0.0039). (C) Nuclear NDRG1 was not associated significantly with metastasis (P= 0.1627). Unpaired tests were conducted to determine the statistical difference for each set of conditions. T1-2 (n=51), T3-4 (n=28), N0 (n= 65), N1 (n= 14), M0 (n= 64) and M1 (n=15).

Table 4. 4 Nuclear and cytoplasmic NDRG1 staining results with clinical data

<table>
<tr><th rowspan="2">Comparison</th><th colspan="3">Nuclear NDRG1 staining</th><th colspan="3">Cytoplasmic NDRG1 staining</th></tr>
<tr><th colspan="2">Results</th><th>p. value</th><th colspan="2">Results</th><th>p. value</th></tr>
<tr><td>Normal vs malignant</td><td colspan="2">Higher in malignant</td><td>0.0002</td><td colspan="2">Lower in malignant</td><td><0.0001</td></tr>
<tr><td rowspan="4">Primary Gleason grade 3.4.&5</td><td rowspan="4">Higher in high Gleason grade</td><td>Anova test</td><td>0.0226</td><td rowspan="4">No statistically significant difference</td><td>Anova test</td><td>0.1742</td></tr>
<tr><td>Grade 4 vs. Grade 3</td><td>0.0395</td><td>Grade 4 vs. Grade 3</td><td>0.30004</td></tr>
<tr><td>Grade 5 vs. Grade 3</td><td>0.0271</td><td>Grade 5 vs. Grade 3</td><td>0.1544</td></tr>
<tr><td>Grade 5 vs. Grade 4</td><td>0.8083</td><td>Grade 5 vs. Grade 4</td><td>0.7377</td></tr>
<tr><td>Stage (T)</td><td colspan="2">Higher in PCa with high stage (T3-T4)</td><td>0.0214</td><td colspan="2">No statistically significant difference</td><td>0.1452</td></tr>
<tr><td>Stage (M)</td><td colspan="2">No statistically significant difference</td><td>0.1627</td><td colspan="2">No statistically significant difference</td><td>0.1313</td></tr>
<tr><td>Stage (N)</td><td colspan="2">Higher in PCa with lymph node invasion (N1)</td><td>0.0039</td><td colspan="2">No statistically significant difference</td><td>0.9412</td></tr>
</table>

In summary, membranous NDRG1 localisation was lower in PCa compared to NP tissues, whereas nucleocytoplasmic localisation was higher. Reduction of membranous and increased nucleocytoplasmic NDRG1 staining was positively associated with increasing primary Gleason grade, but not with clinical stage. In addition, nuclear NDRG1 staining was increased in PCa and was positively associated with increasing primary Gleason grade and clinical stage. In contrast, cytoplasmic NDRG1 staining was decreased significantly in PCa compared to NP, but not associated with other PCa clinical features.

4.2.3.3 NDRG1 localisation in the Bath and TMA cohorts: testing the hypothesis

The results from the two cohorts and if the hypothesis is accepted or rejected is summarised in Table 4.5.

Table 4. 5 The summary of NDRG1 results in the Bath and TMA cohorts.

Parameters	Localisation	Hypothesis	Bath cohort	TMA cohort	Results Confirmed ?	Hypothesis Accepted?
Normal Vs Cancer	Membranous	Lower in PCa	Decrease membranous and increased nucleocytoplasmic in PCa	Decrease membranous and increased nucleocytoplasmic in PCa	YES	YES
	Nuclear	N/A	No significant difference	Higher in PCa	NO	N/A
	Cytoplasmic	Lower in PCa	No significant difference	Lower in PCa	NO	YES
Primary Gleason grades	Membranous	Lower in PCa	No significant difference	Decrease membranous and increased nucleocytoplasmic in high grade.	NO	YES
	Nuclear	N/A	No significant difference	Higher in high grade	NO	N/A
	cytoplasmic	Lower in PCa	No significant difference	No significant difference	NO	NO
Stage T	Membranous	No Significant difference	No significant difference	No significant difference	YES	YES
	Nuclear	N/A	No significant difference	Higher in T3-4	NO	N/A
	cytoplasmic	No Significant difference	No significant difference	No significant difference	YES	YES
Stage M	Membranous	No significant difference	Not tested	No significant difference	N/A	YES
	Nuclear	N/A	Not tested	No significant difference	N/A	N/A
	cytoplasmic	No significant difference	Not tested	No significant difference	N/A	YES
Stage N	Membranous	No significant difference	Not tested	No significant difference	N/A	YES
	Nuclear	N/A	Not tested	Higher in N1	N/A	N/A
	cytoplasmic	No significant difference	Not tested	No significant difference	N/A	YES
Relapsed Vs Non-relapsed	Membranous	Higher in relapse	No significant difference	Not tested	N/A	NO
	Nuclear	N/A	No significant difference	Not tested	N/A	N/A
	cytoplasmic	Higher in relapse	No significant difference	Not tested	N/A	NO

4.2.4 ABCG2

ABCG2 IHC was carried out with the TMA prostate tissue samples and, as with the Bath cohort, the analysis focused on nuclear and cytoplasmic ABCG2 IHC staining.

4.2.4.1 Immunohistochemical staining of ABCG2 in the normal and malignant TMA prostate samples

Nuclear ABCG2 staining was observed in NP tissues and ranged from strong (Figure 4.7 A, arrow) to weak staining or negative (Figure 4.7 B, arrow). Nuclear ABCG2 staining was also found in malignant prostate tissues and the intensity of signal varied widely, ranging from strong (Figure 4.7 C, arrow), moderate (Figure 4.7 D, arrow), weak (Figure 4.7 E, arrow) and negative (Figure 4.7 F&G, arrows). This nuclear staining is not expected for a transmembrane protein, as discussed in chapter 3.

In addition to the nuclear staining, the normal and malignant prostate tissue samples had cytoplasmic ABCG2 staining, with a variable level of staining between cases, ranging from strong (Figure 4.7 A, arrowhead), moderate (Figure 4.7 C, D& E, arrowheads), weak (Figure 4.7 B & F, arrowhead), and negative (Figure 4.7 G, arrow). A negative control (no primary antibody) showed no significant background staining in prostate tissue (Figure 4.7 H, arrow).

4.2.4.2 Association between ABCG2 immunohistochemistry and histopathological parameters of prostate cancer in the TMA cohort

Having carried out IHC staining, the nuclear and cytoplasmic ABCG2 staining was quantified using the proportion and intensity 1 score, as carried out for the Bath cohort samples and then compared between normal vs. malignant prostate tissue, primary Gleason grade, clinical stage. The range of nuclear and cytoplasmic ABCG2 scores varied between 0 to 4 and 0 to 7, respectively (Figure 4.8).

The potential association between ABCG2 IHC results and histopathological parameters of PCa was then analysed and is described below. The first analysis looked at the association between nuclear and cytoplasmic ABCG2 staining in normal vs. malignant prostate tissues. The IHC quantification showed nuclear ABCG2 staining not associated significantly with PCa and other histopathological parameters, including Gleason grade and clinical stage (Figure 4.8 A, C & Table 4.6).

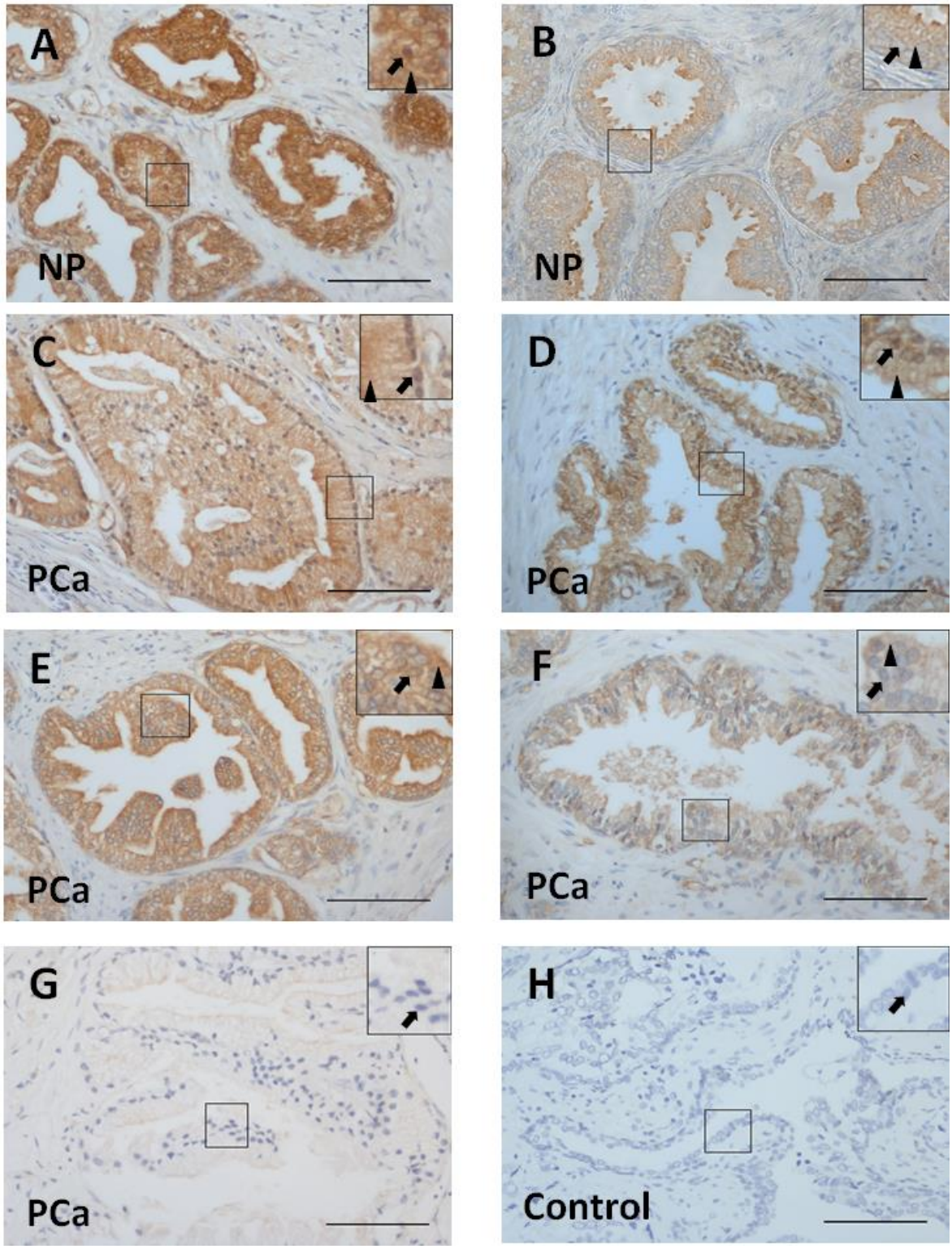

Figure 4. 7 ABCG2 staining in samples from the TMA cohort. ABCG2 was stained heterogeneously in both normal and malignant tissues of the prostate. (A) Strong nuclear (Black arrow) and cytoplasmic (Black arrowhead) ABCG2 staining in NP. (B) Weak cytoplasmic (Black arrowhead) and negative nuclear (Black arrow) ABCG2 staining in NP. (C) Strong nuclear (Black arrow) and moderate cytoplasmic (Black arrowhead) ABCG2 staining in PCa. (D) Moderate nuclear (Black arrow) and cytoplasmic (Black arrowhead) ABCG2 staining in PCa. (E) Weak nuclear (Black arrow) and moderate cytoplasmic (Black arrowhead) ABCG2 in PCa. (F) Weak cytoplasmic (Black arrowhead) and negative nuclear (Black arrow) ABCG2 staining in PCa. (G) No significant staining for ABCG2 (Black arrow) in PCa. (H) Negative control (no primary antibody added) showed no background staining (Black arrow) in PCa. Scale bars=100µm.

In contrast, cytoplasmic ABCG2 staining was significantly reduced in PCa compared to NP tissues (p= < 0.0001) (Figure 4.8 B & Table 4.6). There was a significant difference between cytoplasmic ABCG2 and primary Gleason grade (p= 0.0004) (Figure 4.8 D & Table 4.6). Analysis of IHC data using Tukey's multicomparison tests showed cytoplasmic ABCG2 staining significantly decreased in primary Gleason grade 5 tissues compared to those with a grade 4 (0.0387) or grade 3 (p= 0.0003), and also decreased significantly when comparing between PCa tissues with a grade 4 & 3 (p= 0.0.364) (Figure 4.17 D & Table 4.6). Cytoplasmic ABCGE staining was not associated significantly with clinical stage TNM (Table 4.6).

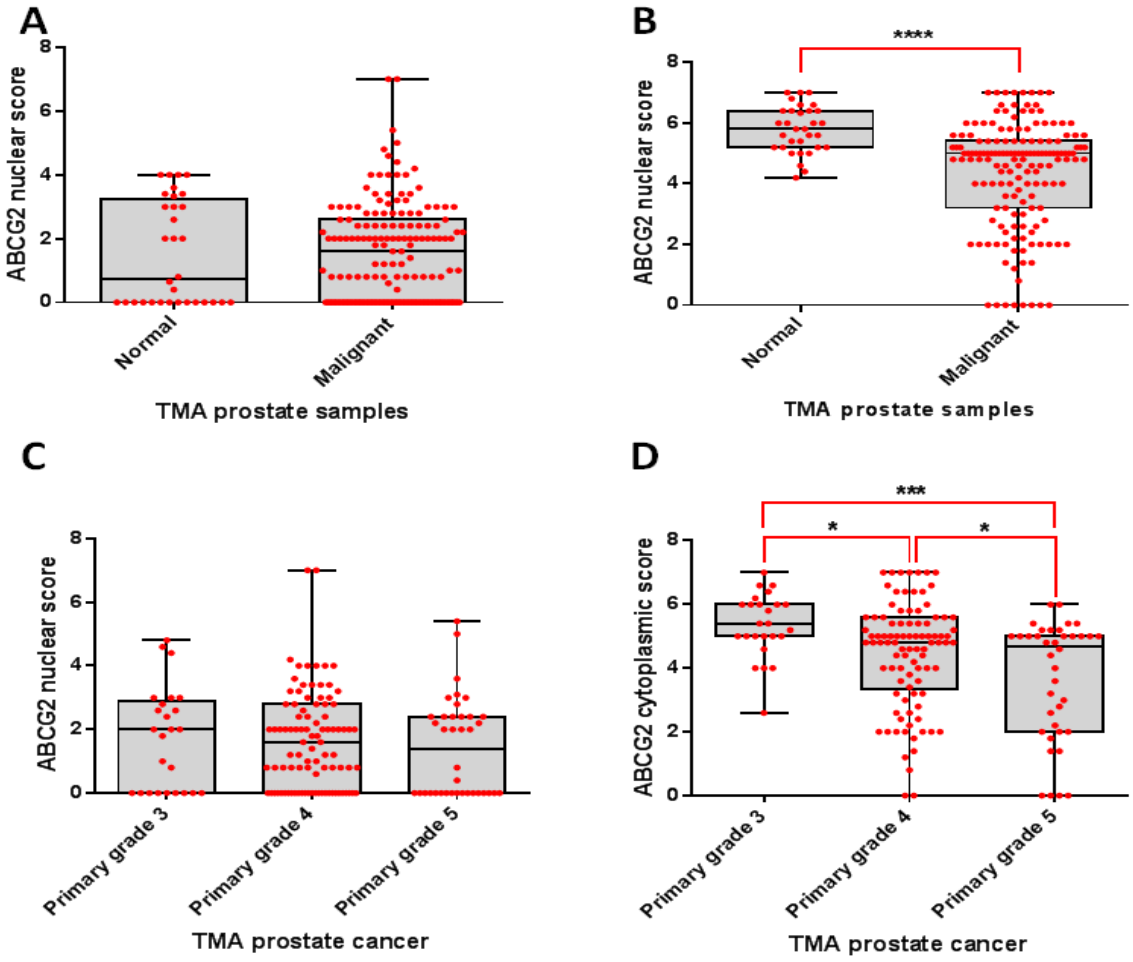

Figure 4. 8 Quantification of nuclear and cytoplasmic ABCG2 staining in the glandular region of normal and malignant TMA prostate tissues. Immunohistochemical staining of ABCG2 was quantified in TMA group using the proportion and intensity 1 score for nuclear and cytoplasmic staining. (A) Nuclear ABCG2 staining showed no significant difference between normal and malignant prostate tissues (p= 0.9141). (B) Cytoplasmic ABCG2 staining was significantly decreased in PCa compared with NP tissues (p=<0.0001). (C) Nuclear ABCG2 nuclear staining was not associated with primary Gleason grade (p= 0.6933). (B) Cytoplasmic ABCG2 staining showed a significant difference among primary Gleason grades (p= 0.0004). Cytoplasmic ABCG2 staining was decreased in Gleason grade 5 compared to grade 4 (p= 0.0387) or grade 3 (p= 0.0003), and also a significant reduction when comparing between grade 4 and 3 (p= 0.0364). Unpaired or one-way ANOVA tests were conducted to determine the statistical difference for each set of conditions. NP (n=16), PCa (n=80), grade 3 (n=13), grade 4 (n=46) and grade 5 (n=18).

Table 4. 6 ABCG2 staining results with clinical data.

Comparison	Nuclear ABCG2 staining			Cytoplasmic ABCG2 staining		
	Results		p. value	Results		p. value
Normal vs malignant	No statistically significant difference		0.9141	lower in malignant		< 0.0001
Primary Gleason grades (3, 4 & 5)	No statistically significant difference	Anova test	0.6933	lower in high Gleason grade	Anova test	0.0004
		Grade 4vs. Grade 3	0.978		Grade 4vs. Grade 3	0.0364
		Grade 5 vs. Grade 3	0.7393		Grade 5 vs. Grade 3	0.0003
		Grade 5 vs. Grade 4	0.7291		Grade 5 vs. Grade 4	0.0387
Stage (T)	No statistically significant difference		0.2507	No statistically significant difference		0.8698
Stage (M)	No statistically significant difference		0.398	No statistically significant difference		0.398
Stage (N)	No statistically significant difference		0.5885	No statistically significant difference		0.3307

In summary, nuclear ABCG2 staining was not associated significantly with PCa and other histopathological parameters. This is consistent with the expectation that it should not be in the nucleus and this staining is likely to be background. In contrast, cytoplasmic ABCG2 staining was decreased significantly in PCa compared to NP and was negatively associated with increasing primary Gleason grade, but not associated with clinical stage TNM.

4.2.4.3 ABCG2 immunostaining in the Bath and TMA cohorts: testing the hypothesis

The results from the two cohorts and if the hypothesis is accepted or rejected is summarised in Table 4.7.

Table 4. 7 The summary of ABCG2 results in the Bath and TMA cohorts

Parameters	Localisation	Hypothesis	Bath	TMA	Results Confirmed?	Hypothesis Accepted?
Normal Vs Cancer	Nuclear	N/A	No significant difference	No significant difference	Yes	N/A
	Cytoplasmic	Lower in PCa	Lower in PCa	Lower in PCa	Yes	Yes
Primary Gleason grades	Nuclear	N/A	higher in high grade	No significant difference	NO	N/A
	Cytoplasmic	No significant difference	No significant difference	Lower in high grade	No	NO
Stage T	Nuclear	N/A	higher in high grade	No significant difference	No	N/A

	Cytoplasmic	No significant difference	No significant difference	No significant difference	YES	YES
Stage M	Nuclear	N/A	Not tested	No significant difference	N/A	N/A
	Cytoplasmic	No significant difference	Not tested	No significant difference	N/A	YES
Stage N	Nuclear	N/A	Not tested	No significant difference	N/A	N/A
	Cytoplasmic	No significant difference	Not tested	No significant difference	N/A	YES
Relapsed vs Non-relapsed	Nuclear	N/A	No significant difference	Not tested	N/A	N/A
	Cytoplasmic	higher in relapse	Lower in relapse	Not tested	N/A	No

4.2.5 ALDH1A1

ALDH1A1 IHC was carried out with the TMA prostate tissue samples and, as with the Bath cohort, the analysis focused on glandular and stromal ALDH1A1 IHC staining.

4.2.5.1 Immunohistochemical staining of ALDH1A1 in normal and malignant TMA prostate samples

ALDH1A1 staining was observed in the glandular and stromal regions of the TMA prostate tissues. Nuclear ALDH1A1 staining was expressed in the glandular regions of the normal and malignant prostate tissues with different levels of staining patterns, ranging from strong (Figure 4.9 B&E arrows) to weak (Figure 4.9 D, arrow) or negative (Figure 4.9 A& G, arrows). Cytoplasmic ALDH1A1 staining was also shown in the glandular regions of normal and malignant prostate tissues, ranging from strong (Figure 4.9 F, arrowhead) to weak (Figure 4.9 E, arrowhead) or negative (Figure 4.9 G). Interestingly, nuclear and cytoplasmic ALDH1A1 staining was found to be restricted in the basal cells of NP, whereas, it was found to be expressed in all PCa epithelial cells, which are mainly luminal, with only a few residual basal cells.

In addition to the glandular ALDH1A1 (nuclear and cytoplasmic) staining, the normal and malignant tissue samples had stromal ALDH1A1 staining, with different staining levels, ranging from strong (Figure 4.9 A & C, arrowheads) to weak (Figure 4.9 G, arrowheads). The negative control was free from background staining on prostate samples (Figure 4. 9 H, arrow).

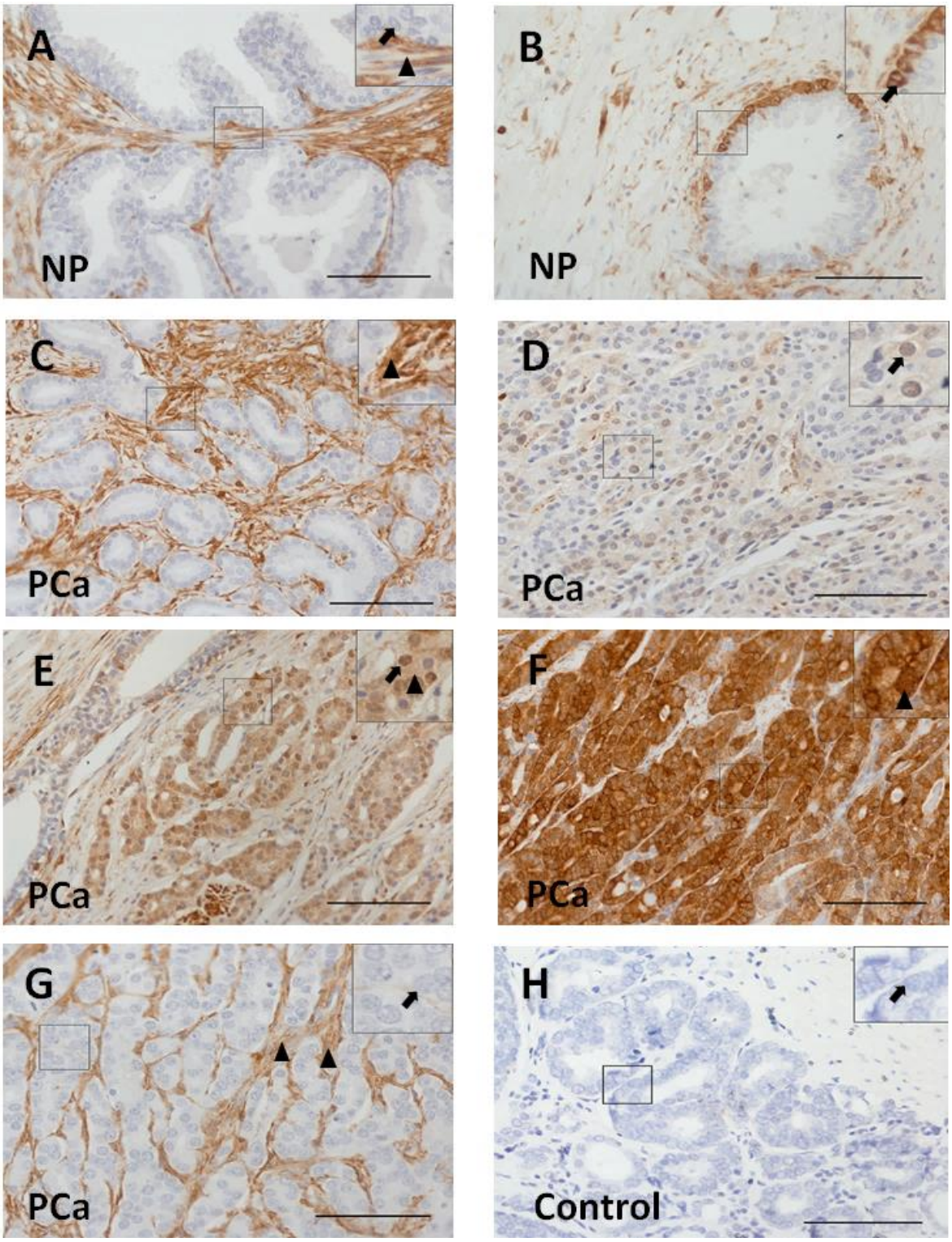

Figure 4. 9 ALDH1A1 was stained heterogeneously in both normal and malignant tissues of the prostate. (A) ALDH1A1 was stained in the stromal region (arrowhead), but not in the glandular region (arrow) of NP. (B) Nuclear AlDH1A1 staining (Black arrow) in the basal cells of NP. (C) Strong stromal (Black arrowhead) with negative glandular ALDH1A1staining in PCa. (D) Weak nuclear ALDHIA1 staining (Black arrow) in the Glandular region of PCa. (E) Strong nuclear (Black arrow) with weak cytoplasmic (Black arrowhead) ALDH1A1 staining in the glandular region of PCa. (F) Strong cytoplasmic ALDH1A1 staining (Black arrowhead) in the glandular region of PCa. (G) Negative ALDH1A1 staining (Black arrow) with weak stromal staining in PCa. (H) The negative control (no primary antibody) showed no staining (Black arrow) in PCa tissue. Scale bars=100µm.

4.2.5.2 Association between ALDH1A1 immunostaining with histopathological parameters of prostate cancer in the TMA cohort

Having carried out IHC staining, nuclear and cytoplasmic ALDH1A1 staining was then quantified using the proportion and intensity 3 scores, whereas stromal proportion and intensity ALDH1A1 staining were quantified using the proportion 1 and intensity 1 respectively, as carried out for the Bath cohort samples. The range of nuclear and cytoplasmic and proportion and intensity stromal ALDH1A1 staining varied between 0-6, 0-6, 0-3, and 0-3 respectively (Figure 4.10). The potential association between ALDH1A1 IHC and histopathological parameters of PCa was then analysed and is described below.

The statistical analysis looked at the ALDH1A1 staining in normal vs. malignant prostate tissues from the TMA cohort. Nuclear ALDH1A1 staining was expressed in 60% of NP and 41% of PCa tissues and the IHC analysis showed nuclear ALDH1A1 significantly increased significantly in PCa compared to NP tissues (p= 0.0451) (Figure 4.10 A & Table 4.8). Cytoplasmic ALDH1A1 staining was also observed in 25% of normal and 41% of malignant prostate tissues. Statistically, there was a significant increase in cytoplasmic ALDH1A1 scores in PCa compared to NP tissues (p= 0.0198) (Figure 4.10 B & Table 4.9). There was no significant association between stromal proportion and intensity ALDH1A1 staining in normal vs. malignant prostate tissues (p= 0.6832 & 0.4775 respectively) (Table 4.9). Both glandular and stromal ALDH1A1 staining was not associated significantly with primary Gleason grade (Tables 4.8 & 4.9).

The statistical analysis showed nuclear and cytoplasmic ALDH1A1 staining significantly increased in advanced stage T (T3-4) compared to localised PCa stage T (T1-2) (P= 0.0011&0.0201 respectively) (Figure 4.10 C&D & Table 4.8). In contrast, nuclear and cytoplasmic ALDH1A1 showed no significant difference between N (N0 vs N1) (p= 0.2337 & 0.9157 respectively) and M (M0 vs M1) (p= 0.0566 & 0.4646 respectively) (Table 4.8). There was also no significant association between Stromal ALDH1A1 staining and clinical stage TNM (Table 4.9).

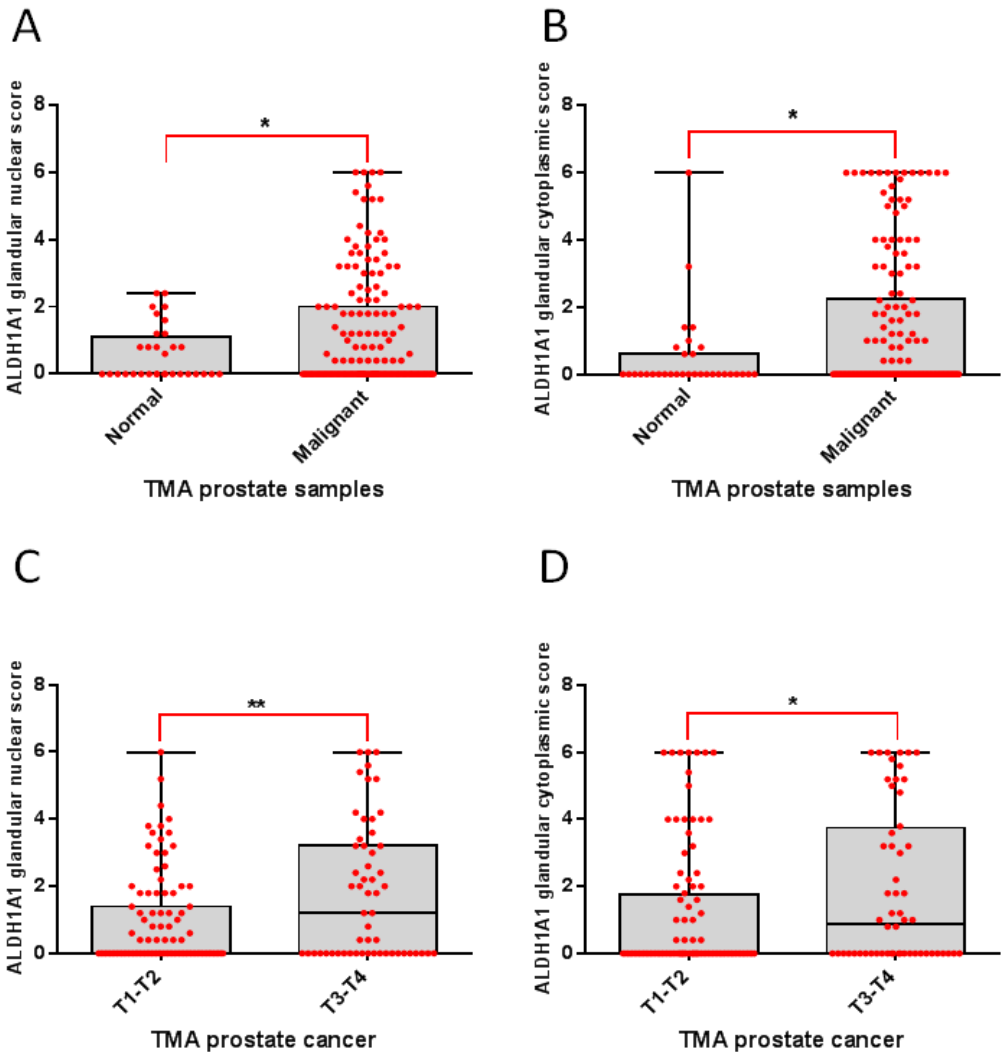

Figure 4. 10 Quantification of nuclear and cytoplasmic ALDH1A1 staining in the glandular region of normal and malignant TMA prostate tissues. IHC staining of ALDH1A1 was quantified in the TMA group using the proportion and intensity 3 scores for nuclear and cytoplasmic IHC staining. (A) Nuclear ALDH1A1 staining was significantly increased in PCa compared with NP tissues (P= 0.0451). (B) Cytoplasmic ALDH1A1 staining was significantly increased in PCa compared with NP tissues (p= 0.0198). (C) Nuclear ALDH1A1 staining was significantly increased in advanced prostate cancer stages T (T3-4) compared to localised PCa T (T1-2) (p= 0.0011). (B) Cytoplasmic ALDH1A1 staining was also significantly increased in advanced PCa stages T (T3-4) compared to localised PCa T (T1-2) (p= 0.0201). Unpaired t-tests were conducted to determine the statistical difference for each set of conditions. NP (n=16), PCa (n=80), T1-2 (n=51) and T3-4 (n=28).

Table 4. 8 Nuclear and cytoplasmic ALDH1A1 staining results with clinical data

Comparison	**Nuclear ALDH1A1 staining**			**Cytoplasmic ALDH1A1 staining**		
	Results	**p. value**		**Results**	**p. value**	
Normal vs malignant	Higher in malignant	0.0451		Higher in malignant	0.0198	
Primary Gleason Grade (3,4 & 5)	No statistically significant difference	Anova test	0.1224	No statistically significant difference	Anova test	0.5533
		Grade 4vs. Grade 3	0.9373		Grade 4vs. Grade 3	0.9598
		Grade 5 vs. Grade 3	0.9995		Grade 5 vs. Grade 3	0.6093

		Grade 5 vs. Grade 4	0.9024		Grade 5 vs. Grade 4	0.608
Stage (T)	Higher in PCa with high stage (T3-T4)		0.0011	Slightly higher in PCa with high stage (T3-T4)		0.0201
Stage (M)	No statistically significant difference		0.0566	No statistically significant difference		0.4646
Stage (N)	No statistically significant difference		0.2337	No statistically significant difference		0.9157s

Table 4. 9 Stromal ALDH1A1 proportion and intensity staining results with clinical data.

Comparison	**ALDH1A1 stromal proportion staining**			**ALDH1A1 stromal intensity staining**		
	Results		**P. value**	**Results**		**P. value**
Normal vs malignant	No statistically significant difference		0.6832	No statistically significant difference		0.4775
Primary Gleason grades (3,4 & 5)	No statistically significant difference	Anova test	0.1276	No statistically significant difference	Anova test	0.0939
		Grade 4vs. Grade 3	0.2236		Grade 4vs. Grade 3	0.2593
		Grade 5 vs. Grade 3	0.115		Grade 5 vs. Grade 3	0.0762
		Grade 5 vs. Grade 4	0.7338		Grade 5 vs. Grade 4	0.5237
Stage (T)	No statistically significant difference		0.1505	No statistically significant difference		0.3552
Stage (M)	No statistically significant difference		0.2182	No statistically significant difference		0.7518
Stage (N)	No statistically significant difference		0.0957	No statistically significant difference		0.7462

In summary, nuclear and cytoplasmic ALDH1A1 staining was increased significantly in PCa compared to NP tissues and was positively associated with advanced PCa stages T (T3-T4 vs T1-T2), but not with primary Gleason grade or other clinical stages (M&N). Stromal ALDH1A1 staining (proportion and intensity) was also not associated with PCa or other parameters such as primary Gleason grade and stage.

4.2.5.3 ALDH1A1 immunostaining in the Bath and TMA cohorts: testing the hypothesis

The results from the two cohorts and if the hypothesis is accepted or rejected is summarised in Table 4.10.

Table 4. 10 The summary of ALDH1A1 results in the Bath and TMA cohorts.

Parameters	localisation	Hypothesis	Bath	TMA	Results Confirmed ?	Hypothesis accepted?
Normal vs Cancer	Glandular Nuclear	N/A	No significant difference	Higher in PCa	NO	N/A
	Glandular Cytoplasmic	Higher in PCa	No significant difference	Higher in PCa	NO	YES
	Stromal Proportion	N/A	No significant difference	No significant difference	Yes	N/A
	Stromal Intensity	N/A	No difference	No difference	Yes	N/A
Primary Gleason grades	Glandular Nuclear	N/A	No significant difference	No significant difference	Yes	N/A
	Glandular Cytoplasmic	Higher in high grade	No significant difference	No significant difference	Yes	NO
	Stromal Proportion	N/A	No significant difference	No significant difference	Yes	N/A
	Stromal Intensity	N/A	No significant difference	No significant difference	Yes	N/A
Stage T	Glandular Nuclear	N/A	No significant difference	Higher in T3-4	No	N/A
	Glandular Cytoplasmic	Higher in T3-4	No significant difference	Higher in T3-4	No	Yes
	Stromal Proportion	N/A	No significant difference	No significant difference	Yes	N/A
	Stromal Intensity	N/A	No significant difference	No significant difference	Yes	N/A
Stage M	Glandular Nuclear	N/A	Not tested	No significant difference	N/A	N/A
	Glandular Cytoplasmic	Higher in M1	Not tested	No significant difference	N/A	NO
	Stromal Proportion	N/A	Not tested	No significant difference	N/A	N/A
	Stromal Intensity	N/A	Not tested	No significant difference	N/A	N/A
Stage N	Glandular Nuclear	N/A	Not tested	No significant difference	N/A	N/A
	Glandular Cytoplasmic	Higher in N1	Not tested	No significant difference	N/A	NO

	Stromal Proportion	N/A	Not tested	No significant difference	N/A	N/A
	Stromal Intensity	N/A	Not tested	No significant difference	N/A	N/A
Relapsed vs Non-relapsed	Glandular Nuclear	N/A	No significant difference	Not tested	N/A	N/A
	Glandular Cytoplasmic	Higher in relapse	No significant difference	Not tested	N/A	NO
	Stromal Proportion	N/A	Lower in relapse	Not tested	N/A	N/A
	Stromal Intensity	N/A	Lower in relapse	Not tested	N/A	N/A

4.2.6 RS1

RS1 IHC was carried out with the TMA prostate tissue samples and, as with the Bath cohort, the analysis focused on nuclear and cytoplasmic RS1 IHC staining.

4.2.6.1 Immunohistochemical staining of RS1 in the normal and malignant TMA prostate samples

The IHC showed nuclear and cytoplasmic staining for RS1 in the TMA prostate tissues. In NP tissues, nuclear RS1 staining was found and ranged from strong (Figure 4.11 A, arrow) to weak (Figure 4.11 B, arrow). Nuclear RS1 staining was also observed in PCa tissues and the intensity of signal varied widely, from strong (Figure 4.11 C, arrow), moderate (Figure 4.11 D, arrow), weak and widespread (Figure 4.11 E, arrow) & negative in some cases (Figure 4.11 G, arrow).

In addition to the nuclear staining, the normal and malignant prostate tissues had cytoplasmic RS1 staining, with a variable level of staining between cases, ranging from strong (Figure 4.11 A, B & C, arrowheads), moderate (Figure 4.11 D, arrowhead) and weak (Figure 4.11 G, arrowheads). A negative control (no primary antibody) showed no significant background staining in PCa tissue (Figure 4.11 H, arrow).

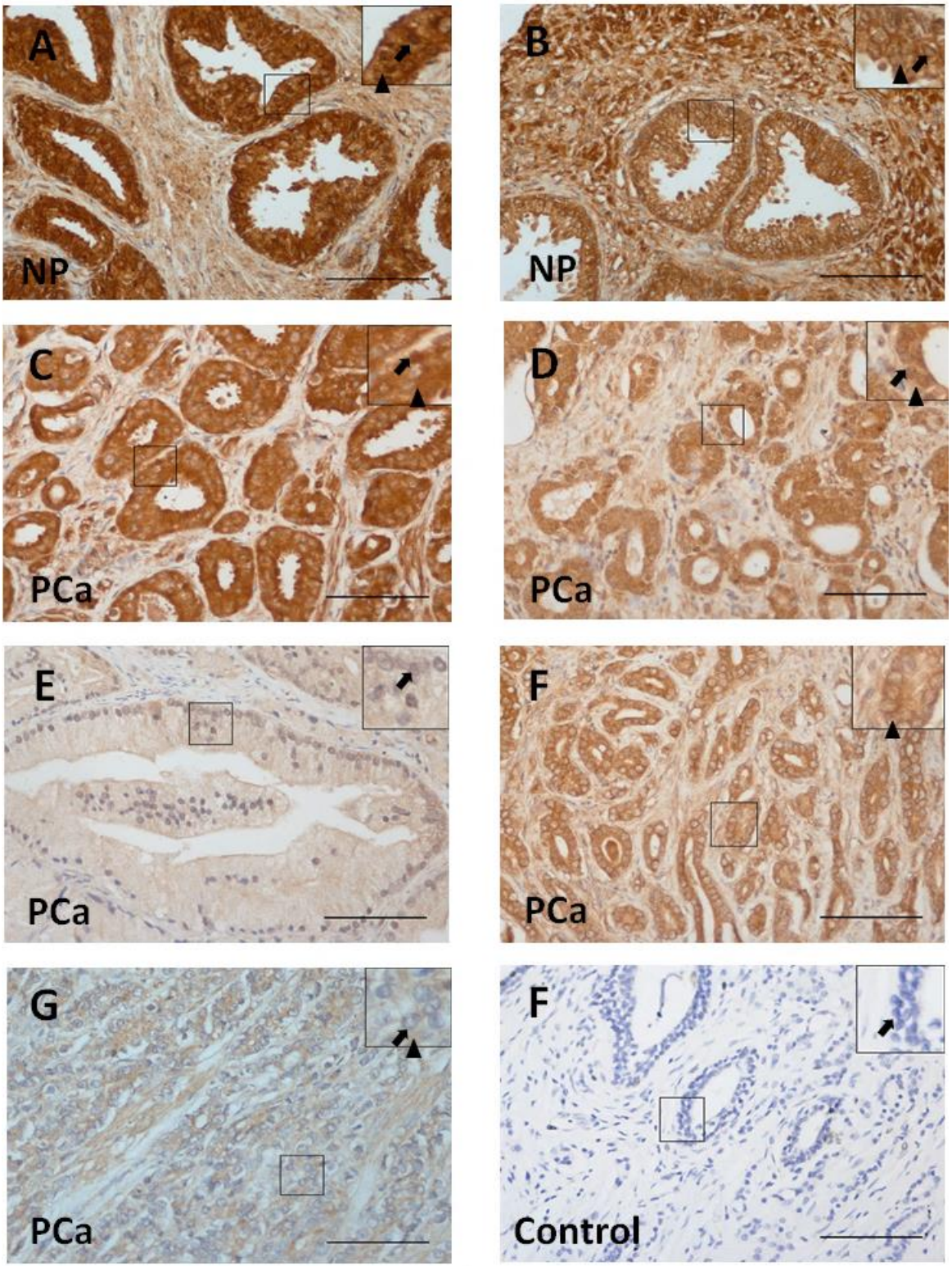

Figure 4. 11 RS1 staining in samples from the TMA cohort. RS1 was stained heterogeneously in both normal and malignant tissues of the prostate. (A) Strong nuclear (Black arrow) and cytoplasmic (Black arrowhead) RS1 staining in NP. (B) Weak nuclear (Black arrow) and strong cytoplasmic (Black arrowhead) RS1 staining in NP. (C) Strong nuclear (Black arrow) and cytoplasmic (Black arrowhead) RS1 staining in PCa. (D) Moderate nuclear (Black arrow) and cytoplasmic (Black arrowhead) RS1 staining in PCa. (E) Weak nuclear (Black arrow) RS1 in PCa. (F) Weak cytoplasmic (Black arrowhead) and negative nuclear RS1 staining in PCa. (G) Negative nuclear (Black arrow) with weak cytoplasmic (Black arrowhead) RS1 staining in PCa. (H) Negative control (no primary antibody added) showed no background staining (Black arrow) in PCa. Scale bars=100µm.

4.2.6.2 Association between RS1 immunostaining and histopathological parameters of prostate cancer in the TMA cohort

Having carried out IHC staining, nuclear and cytoplasmic RS1 staining was then quantified using the proportion and intensity scoring 1 and then compared to histopathological parameters of PCa using the clinical data available for the TMA cohorts. The scoring system for RS1 here is different from the RS1 scoring system used in Chapter 3 because the H score system requires a long time to finish and the signal may be background as a secreted protein RS1 would not be expected to be in the nucleus. For these reasons, our collaborating histopathologist (Dr John Mitchard) suggested using a proportion and intensity score instead (See Chapter 2.3.2). Cytoplasmic staining was also quantified using the same proportion and intensity scoring system. The range of nuclear and cytoplasmic RS1 scores varied between 0-7 (Figure 4.12). The potential association between RS1 IHC results and histopathological parameters of PCa was then analysed and is described below.

The first analysis looked at the RS staining in normal vs. malignant prostate tissues. Quantification of the IHC showed no significant difference between nuclear RS1 staining in normal vs. malignant prostate tissues (p=0.4453) (Figure 4.12 A& Table 4.11). In contrast, cytoplasmic RS1 staining was reduced significantly in PCa compared to NP tissues (p= 0.0088) (Figure 4.12 B& Table 4.11). Nuclear RS1 staining showed a significant difference among primary Gleason grades (p=0.0277) (Figure 4.12 C &Table 4.11). Analysis of the IHC data using Tukey's multicomparison tests showed nuclear RS1 staining increased significantly in primary Gleason grade 5 tissues compared to those with a grade 3 (p= 0.021) (Figure 4.12 A & Table 4.11), whereas, there was no significant difference between nuclear RS1 scores when comparing a primary Gleason grade 4 to grade 3 (p= 0.2184) or grade 5 (p=0.2242) (Figure 4.12 & Table 4.11). Cytoplasmic RS1 showed no significant difference between primary Gleason grade groups (p=0.4295) (Figure 4.12 D & Table 4.11). Nuclear RS1 staining was not associated significantly with the clinical stage (TNM) (Figure 4.12 & Table 4.11). Cytoplasmic RS1 was also associated significantly with lymph node status (N1 vs N0) (P=0.004) (Figure 4.12& Table 4.11), but not with stage T (T1-2 vs T3-4) and M stages (M1 vs M0) (p=0.263 & 0.7687, respectively).

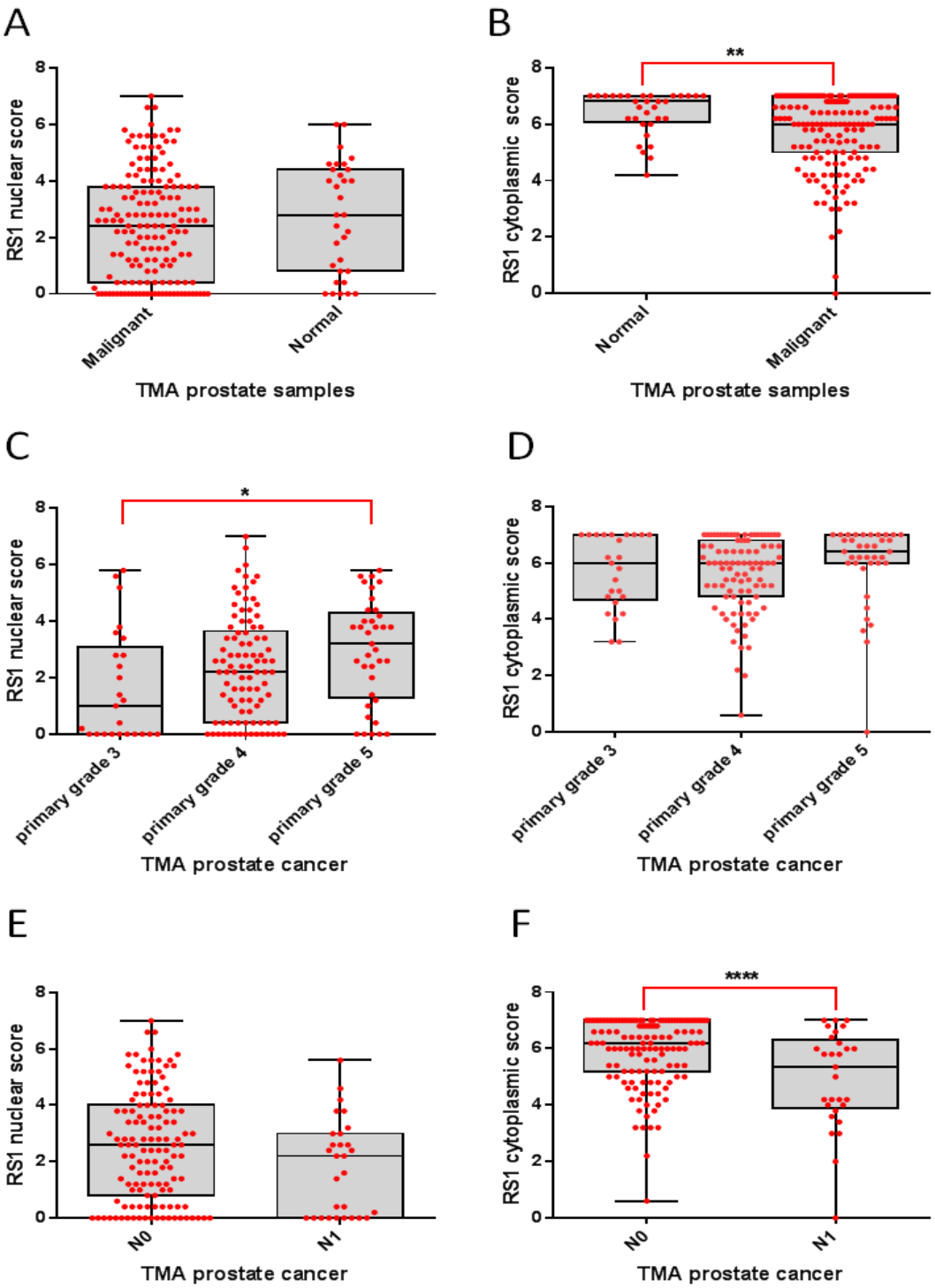

Figure 4. 12 Quantification of nuclear and cytoplasmic RS1 staining in both normal and malignant prostate tissues. Immunohistochemical staining was quantified in the TMA cohort using the proportion and intensity 1 score. (A) Nuclear RS1 showed no significant difference between normal and malignant prostate tissues (p= 0.4453). (B) Cytoplasmic RS1 staining was decreased significantly in PCa compared to NP tissues (p=0.0088). (C) Nuclear RS1 showed a significant difference among primary Gleason grade groups (p= 0.0277). Tukey's multicomparison test showed that nuclear RS1 was significantly associated with increasing primary Gleason grade. (D) Cytoplasmic RS1 staining was not associated with primary Gleason score (p=0.0646). (E) Nuclear ALDH1A1 staining showed no significant difference with stage T (T1-2 ns T3-4) (p= 0.03669). (F) Cytoplasmic RS1 staining was decreased significantly in stage NI compared to N0 cases (p= 0.0004). Unpaired or one-way ANOVA tests were conducted to determine the statistical difference for each set of conditions.NP (n=16), PCa (n=80), grade 3 (n=13), grade 4 (n=46) and grade 5 (n=18), N0 (n= 65) and N1 (n=14).

Table 4. 11 RS1 staining results with clinical data.

Comparison	Rs1 nuclear staining			Rs1 cytoplasmic staining		
	Results		p. value	Results		p. value
Normal vs malignant	No statistically significant difference		0. 4453	Lower in malignant		0.0088
Primary Gleason grades (3,4 & 5)	Higher in high grade	Anova test	0.0277	No statistically significant difference	Anova test	0.4295
		Grade 4vs. Grade 3	0.2184		Grade 4vs. Grade 3	0.9597
		Grade 5 vs. Grade 3	0.021		Grade 5 vs. Grade 3	0.7406
		Grade 5 vs. Grade 4	0.2242		Grade 5 vs. Grade 4	0.3957
Stage (T)	No statistically significant difference		0.3669	No statistically significant difference		0.263
Stage (M)	No statistically significant difference		0.8995	No statistically significant difference		0.7687
Stage (N)	No statistically significant difference		0.0568	Lower in N1		0.0004

In summary, nuclear RS1 staining showed no significant association between normal vs malignant prostate or different primary Gleason grade and stage. In contrast, cytoplasmic RS1 staining was reduced significantly in PCa compared to NP tissues and was negatively associated with lymph node status, but not with primary Gleason grade and clinical stage T&M.

4.2.6.3 RS1 immunostaining in the Bath and TMA cohorts: testing the hypothesis

The results from the two cohorts and if the hypothesis is accepted or rejected is summarised in Table 4.12.

Table 4. 12 The summary of RS1 results in the Bath and TMA cohorts.

Parameters	Localisation	Hypothesis	Bath	TMA	Results Confirmed?	Hypothesis Accepted?
Normal vs Cancer	Nuclear	N/A	No significant difference	No significant difference	Yes	N/A
	Cytoplasmic	No significant difference	No significant difference	Lower in PCa	NO	NO
Primary Gleason grades	Nuclear	N/A	No significant difference	Higher in high grade	NO	N/A
	Cytoplasmic	No significant difference	No significant difference	No significant difference	YES	YES

Stage T	Nuclear	N/A	No significant difference	Higher in high grade	NO	N/A
	Cytoplasmic	No significant difference	No significant difference	No significant difference	YES	YES
Stage M	Nuclear	N/A	Not tested	No significant difference	N/A	N/A
	Cytoplasmic	No significant difference	Not tested	No significant difference	N/A	Yes
Stage N	Nuclear	N/A	Not tested	No significant difference	N/A	N/A
	Cytoplasmic	No significant difference	Not tested	Lower in N1	N/A	NO
Relapsed vs Non-relapsed	Nuclear	N/A	No significant difference	Not tested	N/A	N/A
	Cytoplasmic	Higher in relapse	No significant difference	Not tested	N/A	NO

4.3 Discussion

4.3.1 Summary of the confirmed results

Results presented in this chapter quantify the IHC staining levels of five candidate biomarkers, β-catenin, NDRG1, ABCG2, ALDH1A1 and RS1 in normal vs. malignant prostate tissue, different primary Gleason grades and stages. Previous work presented in Chapter 3 looked at the expression of the same makers in an independent cohort of patients, the Bath cohort. Patterns of expression were accepted as having been confirmed if they were found in both cohorts of patients or were shown in the larger TMA cohort and in the Bath cohort as not significant, but have some trend. These results are summarised in Table 4.13.

In this discussion, I will consider how the staining patterns of these candidate biomarkers compare to previous reports and then describe what the patterns of expression suggest about the possible roles of these proteins in PCa.

Table 4. 13 Confirmed patterns of expression for the candidate biomarkers.

Biomarkers	Confirmed patterns of expression
β -catenin	❖ Nuclear and cytoplasmic β -catenin staining was decreased in PCa and was negatively associated with primary Gleason grade, but not with stage and relapse.
NDRG1	❖ The frequency of membranous localisation was reduced in PCa and was negatively associated with increasing primary Gleason grade. In contrast, nucleocytoplasmic localisation was increased in PCa and high Gleason grade, but not stage and relapse. ❖ Cytoplasmic NDRG1 staining was reduced in PCa, but not with grade, stage and relapse. ❖ Nuclear NDRG1 staining increased in PCa and was positively associated with increasing grade and stages, but not with relapse.
ABCG2	❖ Cytoplasmic ABCG2 staining was reduced in PCa and was negatively associated with primary Gleason grade and relapse, but not with the stage.
ALDH1A1	❖ Nuclear and cytoplasmic ALDH1A1 staining was increased in PCa and was positively associated with stage T (T1-2 vs T3-4). ❖ Stromal ALDH1A1 staining was negatively associated with relapse but not normal vs malignant, grades and stages.
RS1	❖ Changes in nuclear and cytoplasmic RS1 staining was not associated with clinical features of PCa.

4.3.2 β-catenin is decreased significantly in prostate cancer and is negatively associated with increasing primary Gleason grades

Previous studies have reported contradictory β-catenin results in prostate tissue samples. In the current study, a reduction of nuclear β-catenin staining was observed in PCa compared to NP tissues from both cohorts. This data is supported by previous studies with a large cohort (Horvath *et al*., 2005; Whitaker *et al*., 2008), but was inconsistent with Ipekci, *et al* data which is reported increased nuclear β-catenin staining in PCa compared to BPH (Ipekci *et al*., 2015). In addition, cytoplasmic β-catenin staining was decreased in PCa compared to NP tissues, but this reduction was not significant, perhaps due to the small sample size of this cohort. The cytoplasmic data was consistent with a previous finding (Ipekci *et al.,* 2015), but was not supported by other previous literature (Chen *et al.*, 2004; Horvath *et al.*, 2005). The different outcome of the literature may become due to using different antibodies and/or different scoring systems. Taken together, the results suggest that loss β-catenin (nuclear and cytoplasmic) might be an early event in PCa formation.

Nuclear and cytoplasmic β-catenin staining was negatively associated with increasing primary Gleason grade in both cohorts. This data largely agreed with the previous findings

(Aaltomaa *et al.*, 2005; Jaggi *et al.*, 2005; Kallakury *et al.*, 2001b; Whitaker *et al.*, 2008). In contrast, this was not supported by other data (Horvath *et al.*, 2005; Ipekci *et al.*, 2015) which showed no significant association between β-catenin staining and Gleason grade. The use of different scoring systems may explain this difference. The nuclear data is not supported by Jaggi *et al*. study which demonstrated increased nuclear β-catenin localisation with increasing Gleason grade (Jaggi *et al.*, 2005). However, Jaggi *et al*. study had a small number of PCa cases (17) as well as used a different scoring system. Taken together, our results and previous studies suggest that β-catenin may play a role in PCa progression and downregulation of β-catenin may be considered to be a worse indicator for PCa aggressiveness.

In this study, nuclear and cytoplasmic β-catenin staining was not associated with clinical stage except that there was a negative association between cytoplasmic staining and PCa metastasis in the TMA cohort. The association between cytoplasmic β-catenin and metastasis was consistent with (Horvath *et al.*, 2005; Jaggi *et al.*, 2005), but inconsistent with other previous studies (Aaltomaa *et al.*, 2005; Ipekci *et al.*, 2015). In summary, some of the current data suggest that β-catenin reduction may play a role in metastasis, while other studies contradict this conclusion.

There was also no association between β-catenin staining and risk of biochemical recurrence in the Bath cohort data. This was consistent with some previous reports (Kallakury *et al.*, 2001b; Jaggi *et al.*, 2005; Whitaker *et al.*, 2008), but inconsistent with the Horvath *et al* report that found PCa patients with less than 10% positive nuclear β-catenin staining had decreased biochemical relapse-free survival (Horvath *et al*., 2005). Therefore, the majority of evidence suggests there is not an association between β-catenin staining and risk of biochemical recurrence.

Previous studies have proposed different functional roles for β-catenin in cancer, including PCa (Miyabayashi *et al.*, 2007; Miki *et al.*, 2011). However, this section will focus on a couple of possible mechanisms for β-catenin which would be consistent with its role in PCa based on the expression data described above. Increased β-catenin levels can induce apoptosis in both normal and malignant cells independently of TCF transcriptional activity and/ or cell cycle/apoptosis regulators, including p53 and transcription factor E2F1(alternatively named retinoblastoma-associated protein 1 or retinoblastoma-binding protein 3) (Kim *et al*., 2000), suggesting that β-catenin might interact with other proteins through a

potential death domain to induce apoptosis. Decreased β-catenin might then protect cells from apoptosis. Other studies have reported that β-catenin can bind to the transcription factor TCF/LEF, which is located in the nucleus, and/or p300 (Histone acetyltransferase p300) to form a β-catenin/TCF/ P300 complex which plays a role to induce differentiation (Miki *et al*., 2011; Miyabayashi *et al*., 2007), suggesting reduction of β-catenin in cells may lead to reduced differentiation and promotion of PCa.

Taken together, it appears that reduced β-catenin signalling is involved in PCa tumorigenesis and decreased β-catenin levels may be a promising marker of a worse prognosis in localised PCa. However, further study is needed to confirm the staining patterns of β-catenin in prostate tissue samples, using either a second independent antibody and/or mRNA probe (RNAscope®) which can detect the mRNA level in prostate tissues from both cohorts. Finally, it will be very interesting to address the functional role of β-catenin in PCa formation and progression, using tissue culture (see Chapter 8 for more details).

4.3.3 Nuclear NDRG1 is increased in prostate cancer and is positively associated with increasing grade and stage, while membranous and cytoplasmic NDRG1 staining is decreased in prostate cancer

There are a number of studies focused on either NDRG1 localisation or staining in normal and malignant prostate. However, these studies come with contradictory results. IHC results in both cohorts showed that decreased membranous localisation of NDRG1 was observed in PCa compared to NP and was positively associated with increasing primary Gleason grade, but not with stage and replace. In contrast, nucleocytoplasmic localisation was increased significantly in PCa and was positively associated with increasing primary Gleason grade, but not with stage and relapse. The association between NDRG1 localisation and Gleason grade in the Bath cohort was not significant, which may be because of the small sample size. However, this data is consistent with the previous findings (Caruso *et al.*, 2004; Song *et al.*, 2010; Symes *et al.*, 2013), suggesting that the change in NDRG1 localisation might play a role in PCa formation and differentiation.

In addition, this study is the first to quantify nuclear NDRG1 staining and shows an association between nuclear NDRG1 staining in normal vs malignant, different primary Gleason grades, clinical stages and relapse. The IHC data from the TMA cohort shows that nuclear NDRG1 staining was increased significantly in PCa compared to NP tissues and was

positively associated with increasing primary Gleason grade and clinical stage. However, the nuclear NDRG1 staining in the Bath cohort was not significant, perhaps due to the small sample size in this cohort. However, our data was supported by Song *et al.* who reported that nuclear NDRG1 expression was increased in colorectal carcinoma compared to normal tissues (Song *et al*., 2013) as well by Hosoya *et al.* who reported increased nuclear NDRG1 was positively associated with grade and stage of renal cell carcinoma (Hosoya *et al*., 2013), suggesting that increased nuclear NDRG1 expression plays an important role in PCa formation and progression and could be a predictive marker for a poor prognosis.

Cytoplasmic NDRG1 staining was decreased in malignant prostate tissues compared to NP tissues from the TMA cohort, but not the Bath cohort. This result was largely agreed with a previous finding (Li *et al.*, 2015b), suggesting that reduction cytoplasmic NDRG1 staining might play a role in PCa formation. However, this data was contradictory with (Symes *et al*., 2013; Song *et al*., 2010). Cytoplasmic NDRG1 staining was not associated significantly with primary Gleason grade, stage and relapse. This data is consistent with a previous prostate report (Symes *et al.*, 2013), but not with the other data (Bandyopadhyay *et al.*, 2003; Song *et al.*, 2010). This difference between the literature could be because of the sample size, the use of different antibodies and/or the use of different methods. Taken together, a reduction in cytoplasmic NDRG1 level, in addition to the increase in nuclear NDRG1 described above, could play a role in PCa formation and progression.

What role might NDRG1 play in cancer formation? Previous studies have been found either an oncogenic or tumour suppressor role of NDRG1 in cancer. However, the majority of the studies have reported a tumour suppressor role, including for PCa (Bae *et al.*, 2013; Song *et al.*, 2010). There are many different possible mechanisms to explain this role in cancer. First, NDRG1 upregulates frequently rearranged in advanced T cell protein (FRAT1) which plays an important role in preventing binding of Glycogen synthase kinase 3 beta (GSK3B) with the distraction complex, that subsequently prevents phosphorylation of β-catenin and then prevents nuclear translocation of phosphorylated β-catenin (Jin *et al.*, 2014). This study also found that blocking nuclear β-catenin translocation may occur through NDRG1's role to inactivate P21- activated kinase 4 (PAK4) protein, which plays a transporter role for β-catenin. Therefore, loss of cytoplasmic NDRG1 could increase Wnt signalling (Jin *et al.*, 2014), which is consistent with the β-catenin data described above. In addition, a previous study found that increased NDRG1 in pancreatic cancer cell lines inhibits PI3K and Ras signalling pathways through increasing PTEN and SMAD4 which inhibit tumour

progression (Kovacevic *et al.*, 2013), providing another mechanism to explain its tumour suppressor role.

In addition to a tumour suppressor role, previous literature has reported an oncogenic role for NDRG1. Increased NDRG1 may promote the growth of tumours through its role in promoting metastasis and angiogenesis, as well as protecting cells from apoptosis (Wei et al., 2013). A study done by Ai *et al* reported that increased NDRG1 expression may promote oesophagal squamous cell carcinoma progression through modulating Wnt signalling pathway by affecting transducin-like enhancer of split 2 (TLE2) and β-catenin (Ai *et al.*, 2016). This is confirmed by our data which found a moderate negative correlation between nuclear staining of β-catenin and nuclear NDRG1 (R=-0.56) (data are not shown), suggesting that increased nuclear NDRG1 reduces nuclear β-catenin levels. In colorectal carcinoma, NDRG1 translocation from the membrane to the nucleus of cells may play a role in lymph node metastasis by regulation of E-Cadherin expression (Song *et al*., 2013). This is confirmed by our data which found increased nuclear NDRG1 significantly associated with lymph node metastasis in the TMA cohort.

Taken together, this study suggests that decreased membranous and cytoplasmic NDRG1 levels in PCa cells may downregulate PTEN and activate PI3K signalling pathways which lead to increased proliferation and inhibit apoptosis. Whereas increased nuclear localisation of NDRG1 may lead to a decrease in nuclear β-catenin transcriptional activity, however, this is complicated as a reduction in cytoplasmic may have the opposite effect. The change of NDRG1 localisation and/or increased nuclear NDRG1 level could be a poor indicator for PCa prognosis and it could be a useful prognostic biomarker for PCa. Further studies are needed to confirm the staining patterns of NDRG1 using either IHC with a second independent antibody or RNAscope to detect the mRNA level of NRGG1 in prostate tissue from both cohorts. In addition, It would be very interesting to be to use a large cohort with clinical data about a risk of relapse to confirm if there is an association between NDRG1 staining and biochemical relapse. Future research is also needed to determine if there is an association between NDRG1 localisation and PTEN level in PCa. Finally, it would also be interesting to investigate NDRG1 functional roles in PCa using an *in vitro* cell culture approach (see final discussion for more details).

4.3.4 Cytoplasmic ABCG2 expression is reduced in prostate cancer and is negatively associated with increasing Gleason grades and relapse

Cytoplasmic ABCG2 staining was reduced significantly in PCa compared to NP tissues. This data was supported by the literature (Thompson *et al.*, 2008) and taken together suggests that decreased cytoplasmic ABCG2 may play an important role in PCa formation. However, this data disagrees with the previous ABCG2 mRNA data that showed there was no significant association in ABCG2 mRNA level between normal and malignant using real-time PCR (Guzel *et al.*, 2014), suggesting there might be differences between mRNA and protein expression. It might also be that the difference is caused by using different methods for detection. Cytoplasmic ABCG2 staining was significantly reduced with increasing primary Gleason grade. This data is supported by Castellon *et al* data who reported that PCa tissues with a medium Gleason grade showed increased ABCG2 staining compared to those with a high Gleason grade (Castellon *et al*., 2012). Cytoplasmic ABCG2 staining was not associated with clinical stage, which is consistent with a previous finding on breast cancer (Xiang *et al.*, 2011). Data from the Bath cohort showed a significant reduction of cytoplasmic ABCG2 staining in relapsed tissues compared to those with non-relapsed, suggesting ABCG2 may play a role to prevent PCa relapse. This result is also contradictory with previous ABCG2 mRNA data (Guzel *et al*., 2014; Pfeiffer *et al*., 2011).

This study showed nuclear ABCG2 staining in prostate samples for the first time. The statistical analysis showed that there was no significant difference between nuclear ABCG2 staining when comparing NP tissues to those with malignancies. The Bath data shows that there was a positive association between nuclear ABCG2 staining and Gleason grade or clinical stage, but not with relapse. In contrast, the TMA data shows that nuclear ABCG2 was not associated with primary Gleason grade and clinical stage. ABCG2 as a transmembranous transporter protein is not expected to be expressed in the nuclei of cells, but two different studies have reported the nuclear localisation of ABCG2 in glioblastoma multiform cells (Bhatia *et al.*, 2012) and lung cancer cells (Liang *et al.*, 2015) using different IHC and immunofluorescence. This might suggest a role for ABCG2 in the nucleus, but another possibility is that the nuclear ABCG2 staining is non-specific. Further study is needed to confirm if there is nuclear localisation of ABCG2 in prostate tissues.

Previous studies have proposed different roles for ABCG2 in human tissues, including cancer. The ABC transporter superfamily proteins use ATP hydrolysis to transport different molecules through the plasma membrane, including drugs (Fetsch et al., 2006). ABCG2 is found to play a role in transporting cytotoxic anti-cancer agents, as well as endogenous materials out of cells (Gottesman *et al.*, 2002). A previous ABCG2 review reported that by active efflux of anticancer medicine, the expression of ABCG2 may directly cause multidrug resistance (Nakanishi and Ross, 2012). However, ABCG2 may also play additional roles in promoting proliferation and maintaining the undifferentiated phenotype of SCs (Ding *et al*., 2010). A more recent study done by Sabnis *et al*. reported that ABCG2 has a role in the maintenance of PSCs as well as in providing a rationale for targeting ABCG2 for differentiation therapy in PCa, using cancerous and non-cancerous prostate cell lines (Sabnis *et al.*, 2017). This role might occur because inhibiting ABCG2-mediated androgen efflux with ABCG2 inhibitor (Ko143) increases the nuclear translocation of AR (Sabnis *et al*., 2017). A third study has identified that androgen-independent PCa patients with the Q141K variant have a shorter overall survival time than patients without the single nucleotide polymorphism (SNP) (Gardner et al., 2008), suggesting that the reduced function of the variant ABCG2 protein helps to increase the intracellular concentration of androgen which may play a role in driving the proliferation of the cells. This last study appears most consistent with the loss of expression described here.

Data presented here suggest that downregulation of ABCG2 may play an important role in cancer formation and progression as well as increasing the risk of relapse. ABCG2 in the nucleus may have another role, but this role is still unclear. Further study is needed to confirm the nuclear localisation of ABCG2, perhaps using immunofluorescence or nuclear fractionation. In addition, it will very important to confirm the cytoplasmic staining patterns of ABCG2 in prostate tissues, using a second independent antibody (IHC) or mRNA probe (RNAscope®) in normal and malignant prostate tissues from both cohorts. The future work could be carried out with a large cohort, which has clinical information about a risk of biochemical recurrence of PCa to assess the role of ABCG2 in risk of recurrence. Finally, it would also be interesting to investigate NDRG1 functional roles in PCa using an *in vitro* cell culture approach (see final discussion for more details).

4.3.5 Glandular ALDH1A1 is increased in prostate cancer and is positively associated with tumour size, whereas, stromal ALDH1A1 is negatively associated with relapse

IHC demonstrated glandular and stromal staining for ALDH1A1 in both normal and malignant prostate tissues from the Bath cohort. This was consistent with a previous finding (Deng *et al.*, 2010; Matsika *et al.*, 2015). ALDH1A1 staining was found to be restricted to basal cells of NP tissues, but was expressed all epithelial cells of PCa tissues.

Nuclear and cytoplasmic ALDH1A1 staining was increased significantly in PCa compared to NP and was positively associated with clinical stage T in the TMA cohort. The results largely agreed with (Ting *et al.*, 2009; Kalantari *et al.*, 2017; Matsika *et al.*, 2015), suggesting increased ALDH1A1 may play a role in PCa formation and progression. The results were inconsistent with the Bath data, but this may be because of the sample size. Nuclear and cytoplasmic ALDH1A1 staining was not associated significantly with primary Gleason grade and metastasis in both cohorts. This results are consistent with a previous gastric cancer study (Li *et al.*, 2014), but was inconsistent with other PCa studies (Ting *et al.*, 2009; Kalantari *et al.*, 2017). The cause of this difference is not known, but might be explained as the previous prostate studies (Ting *et al.*, 2009; Kalantari *et al.*, 2017) used different antibody concentrations and IHC staining protocols. It might also be that the difference is caused by differences in the patient populations. The current study shows for the first time that the stromal ALDH1A1 staining was not associated significantly with PCa and other histopathological parameters, including primary Gleason grade and clinical stage. The Bath data, however, shows for the first time that stromal ALDH1A1 was negatively associated with biochemical relapse, suggesting loss of stromal staining of ALDH1A1 might play a role in relapse.

ALDHIA1 has been linked to the SC population and is thought to be a marker of SCs and CSCs (Tomita *et al*., 2016). Other work reported that ALDH1A1 plays an important role in catalyzing the oxidation of retinal to retinoic acid (RA) that is linked to differentiation during development of cells (Tomita *et al.*, 2016; Ting *et al*., 2009). Increased ALDH1A1 in cells could cause RA to accumulate in the nucleus and then either bind with RA receptor RAR and/ or retinoid X receptors (RXRS) to increase RARβ genes. These play a role in differentiation, apoptosis and growth inhibition and/ or increases c-Myc and Cyclin D1 transcription which can promote cell proliferation, anti-apoptosis and tumour growth in CSCs (Tomita *et al*., 2016). Therefore, increased ALDH1A1 could lead to increased cell

proliferation and tumour growth through its role in increasing RA levels. Further study is needed to confirm the staining patterns of ALDH1A1 in prostate tissues, using a second independent antibody in normal and malignant prostate tissues from both cohorts. The future work could be carried out with a large cohort, which has clinical information about a risk of biochemical recurrence of PCa to assess the role of ALDH1A1 in risk of recurrence.

4.3.6 Changes in nuclear and cytoplasmic RS1 staining was not associated with clinical features of prostate cancer

IHC data showed nuclear staining was not associated with the clinical feature of PCa in both cohorts. This result was consistent with the previous finding (Sharpe, 2016). RS1 is an extracellular protein and is not expected to be in the nucleus of prostate gland cells. This staining could be non-specific.

Cytoplasmic RS1 was decreased significantly in PCa compared to NP tissues from the TMA, whereas, there was no association in cytoplasmic RS1 staining between normal vs malignant prostate tissues from the Bath cohort. This difference may be because of the sample size or using different scoring systems. There was no significant association between cytoplasmic RS1 staining and primary Gleason grade, clinical stage and relapse. This result was consistent with previous findings from our laboratory (Sharpe, 2016). The TMA data showed that cytoplasmic RS1 was negatively associated with clinical stage (N). This data, however, was inconsistent with previous bioinformatic data, cBioportal (http://www.cbioportal.org/) that found RS1 amplification was observed more frequently in a subset of metastatic and castration-resistant prostate carcinomas, using 10 large prostate cancer microarray datasets.

In summary, there is little evidence to suggest a link between expression of RS1 and PCa formation or progression, although there remains a possible role for cytoplasmic expression that could be studied further.

4.3.7 Conclusion

In conclusion, decreased β-catenin, ABCG2, membranous and cytoplasmic NDRG1 staining is observed in PCa compared to NP tissues, whereas, nuclear NDRG1 and glandular ALDH1A1 staining is increased in PCa compared to NP. β-catenin and cytoplasmic ABCG2 staining are negatively associated with increasing Gleason grade, whereas nuclear NDRG1 staining is positively associated with increasing primary Gleason grade. In addition,

increased nuclear NDRG1 staining is positively associated with clinical stage and increased ALH1A1 staining was only associated with stage T. In contrast, decreased cytoplasmic β-catenin and RS1 staining is associated with a lymph node metastasis. ABCG2 and stromal ALDH1A1 staining is only negatively associated with biochemical relapse.

β-catenin, ABCG2 and NDRG1 might play a role in PCa formation and progression and could be prognostic biomarkers for PCa. ABCG2 and stromal ALDH1A1 may have a role to reduce the risk of relapse. However, further studies are needed to confirm the staining patterns of the candidate biomarkers using either a second independent antibody or RNAscope to detect the mRNA levels in prostate tissues from both cohorts. In addition, it would be very interesting to use an academic TMA cohort (Rubin *et al.*, 2002; Nariculam *et al.*, 2009; Symes *et al.*, 2013) with clinical information to determine if there is an association between these biomarkers staining and biochemical relapse. Additional future work is considered in the final discussion.

CHAPTER FIVE

IDENTIFICATION AND ASSESSMENT OF SOX7 EXPRESSION IN PROSTATE TISSUES FROM THE BATH AND TMA COHORTS

5. Identification and Assessment of Sox7 expression in prostate tissues from the Bath and TMA cohorts

5.1 Introduction

Sex-determining region Y box 7 (Sox7) is one of the candidate biomarkers that was identified for analysis. This chapter focuses on Sox7 and describes why the protein was selected for analysis and presents all the experimental data that was collected for the protein.

5.1.1 Identification of Sox7 using literature searching

Sox7 is a transcription factor that plays an essential role in regulating different biological processes, including haematopoiesis, vasculogenesis and cardiogenesis through embryonic development (Stovall *et al.*, 2014). It is also thought to have a tumour suppressor role in a range of cancers (Cui *et al.*, 2014). Interestingly for this study, reduction of Sox7 levels has been suggested to play a role in PCa formation, progression and relapse (described below and summarised in Table 5.1).

There are several publications that have studied the expression levels of Sox7 in both normal and malignant tissues. Generally, most of these studies reported that nuclear and cytoplasmic Sox7 expression is significantly reduced in cancer tissues compared to normal tissues, including breast (Liu *et al.*, 2016), ovarian (Liu *et al.*, 2014), liver (Wang *et al.*, 2014a; Wang *et al.*, 2017), pancreas (Wang *et al.*, 2017) and prostate (Guo *et al.*, 2008; Zhong *et al.*, 2012). Therefore, these previous studies, especially (Guo *et al.*, 2008; Zhong *et al.*, 2012) provide significant evidence to suggest that the reduction of Sox7 expression may be linked to PCa formation.

In terms of an association between Sox7 expression and histopathological parameters of cancer including grades and stages, previous studies have found that there was no association between Sox7 mRNA and/ or protein expressions and cancer grades in ovarian (Liu *et al.*, 2014), liver (Wang *et al.*, 2014a; Wang *et al.*, 2017) and prostate (Zhong *et al.*, 2012). In contrast, more recent studies showed that Sox7 was significantly reduced with increasing grades of the breast (Liu *et al.*, 2016) and pancreatic cancers (Wang *et al.*, 2017).

Several studies have shown a significant association between Sox7 expression and cancer stages. For example, Sox7 was significantly reduced in an advanced stage of tumours such as ovarian (Liu *et al.*, 2014), liver (Wang *et al.*, 2017) and breast cancers (Liu *et al.*, 2016).

Another IHC study showed that Sox7 mRNA level in PCa patients with a low serum level of PSA and no-metastasis was significantly higher than those with a high serum PSA level and metastasis (Zhong *et al.*, 2012). In contrast, Sox7 expression was not found to be associated with a stage of liver cancer (Wang *et al.*, 2017). Therefore, the more recent studies on the breast (Liu *et al.*, 2016) and pancreatic cancer tissues (Wang *et al.*, 2017) provide significant evidence to suggest that reduction of Sox7 expression is linked to PCa grades and stages.

In terms of a link to PCa relapse, one study has reported that reduction of Sox7 at mRNA level is significantly associated with PCa recurrence (Zhong *et al.*, 2012). However, a link between Sox7 protein level and PCa relapse using IHC has not been described before. Therefore, the association between mRNA Sox7 level and PCa provides evidence to suggest that alterations in Sox7 protein expression could be linked to PCa relapse, but further analysis is required to establish if such a relationship exists.

Given the conflicting evidence, described above, it was decided that it would be worthwhile to re-examine the expression of Sox7 in PCa. Specifically, to examine nuclear and cytoplasmic Sox7 expression in normal and malignant prostate tissues, primary Gleason grade, clinical stage and biochemical recurrence. This would increase our understanding of Sox7 expression and its relationship to PCa formation and clinical parameters, including grades and stages. It would also be the first study to look at the relationship between Sox7 protein expression and relapse using IHC. The hypothesis used predicted that nuclear and cytoplasmic Sox7 expressions will be negatively associated with PCa, primary Gleason grade, clinical stages and biochemical recurrence.

Table 5. 1 Summary of Sox7 key findings and hypothesis

Name & type of Protein	Key publications	Hypothesis
Sox7: A transcription factor	❖ Sox7 protein and mRNA levels were significantly decreased in PCa compared to NP tissues (Guo *et al.*, 2008; Zhong *et al.*, 2012). The reduction in metastatic PCa cases was significantly higher than in non-metastatic cases and was associated with biochemical relapse. Sox7 mRNA level was not associated significantly with Gleason grades (Zhong *et al*., 2012). ❖ A significant reduction of Sox7 expression has been shown in ovarian cancer compared to the normal ovary and this reduction was significantly associated with advanced stages, but not with grades (Liu *et al*., 2014).	❖ Nuclear and cytoplasmic Sox7 staining will be reduced in PCa compared to NP tissues. ❖ Nuclear and cytoplasmic Sox7 staining will be negatively associated with primary Gleason grade, clinical

	❖ Nuclear and cytoplasmic Sox7 expression was reduced in breast cancer compared to normal breast tissues and was negatively associated with grades and stages (Liu *et al*., 2016). ❖ Sox7 was decreased significantly in liver and pancreatic cancers compared to benign tissues. In liver cancer, this reduction was significantly associated with advanced stages, but not associated with grades. In contrast, this reduction in pancreatic cancer was significantly associated with increasing grades, but not with stages (Wang *et al*., 2017).	stage and biochemical relapse.

5.1.2 Aims

1. To determine the expression patterns of Sox7 in normal and malignant prostate tissues from the Bath and the TMA cohort patients.
2. To establish if Sox7 expression correlates with clinical features of PCa, including primary Gleason grade, clinical stage and biochemical recurrence.
3. To confirm the specificity of Sox7 staining pattern and IHC results by repeating the experiments with a second independent antibody.

5.2 Results

5.2.1 Testing of antigen retrieval methods

Having chosen Sox7 as a candidate biomarker, the next step was to assess its expression using IHC. Before this could be carried out, different types of antigen retrieval such as citrate or Tris/EDTA buffers were tested to establish which give the best staining for Sox7. The staining was carried out with an anti-Sox7 antibody from Abcam that is referred to here as the anti-Sox7 A antibody. IHC staining with citrate and Tris/ EDTA buffers showed nuclear and cytoplasmic anti-Sox7 A staining in prostate tissues from the Bath cohort. However, this study chose citrate buffer as antigen retrieval for Sox7 because the nuclear and cytoplasmic anti-Sox7 A staining patterns in the citrate buffer (Figure 5.1 A) was clearer than in Tris/EDTA (Figure 5.1 B).

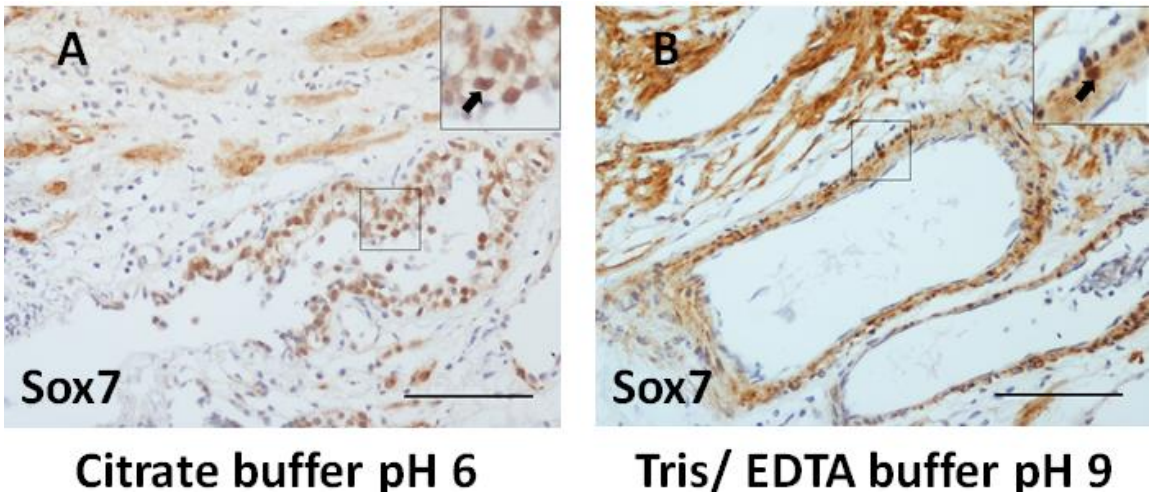

Figure 5. 1 Expression of Sox7 after different antigen retrieval methods. (A) Strong nuclear (Black arrow) and weak cytoplasmic anti-Sox7 A staining was observed in prostate tissues from the Bath cohort, using citrate buffer (B) Strong nuclear (Black arrow) and cytoplasmic anti-Sox7 A staining was observed in prostate tissue, using Tris/EDTA buffer. Sox7 showed a clearer nuclear and cytoplasmic staining with citrate compared to Tris/ EDTA buffers. Scale bars=100µm with inserts at 2x zoom.

5.2.2 Expression of Sox7 in the Bath cohort using the anti-Sox7 A antibody

To test the hypothesis, that nuclear and cytoplasmic Sox7 staining will be decreased in PCa and will be negatively associated with primary Gleason grade, clinical stage and biochemical recurrence. IHC was carried out with the Bath cohort prostate tissue samples using the anti-Sox7 A antibody.

5.2.2.1 Immunohistochemical expression of anti-Sox7 A in the normal and malignant Bath prostate samples

In NP tissues, nuclear anti-Sox7 A staining was found and ranged from strong (Figure 5.2 A, arrow) to weak (Figure 5.2 B, arrow). Nuclear anti-Sox7 A staining was also observed in PCa tissues and the intensity of signal varied widely, from a strong and widespread (Figure 5.2 C, arrow) to a weak and scattered (Figure 5.2 E, arrow) or a negative (Figure 5.2 F, arrow).

In addition to the nuclear staining, cytoplasmic anti-Sox7 A was also observed in the glandular regions of the Bath prostate tissues with variable staining levels, ranging from strong (Figure 5.2 D, arrowhead), weak (Figure 5.2 A& E, arrowheads) and negative (Figure

5.2 F, arrow). Sox7 is expressed in the cytoplasm of normal liver tissues (Wang *et al.*, 2017) and so this study used normal liver tissue as a positive control for Sox7 expression and as expected IHC staining showed cytoplasmic anti-Sox7 A staining in normal liver tissue (Figure 5.2 G, arrowhead). A negative control showed no significant background staining in prostate tissue (Figure 5.2 H, arrow).

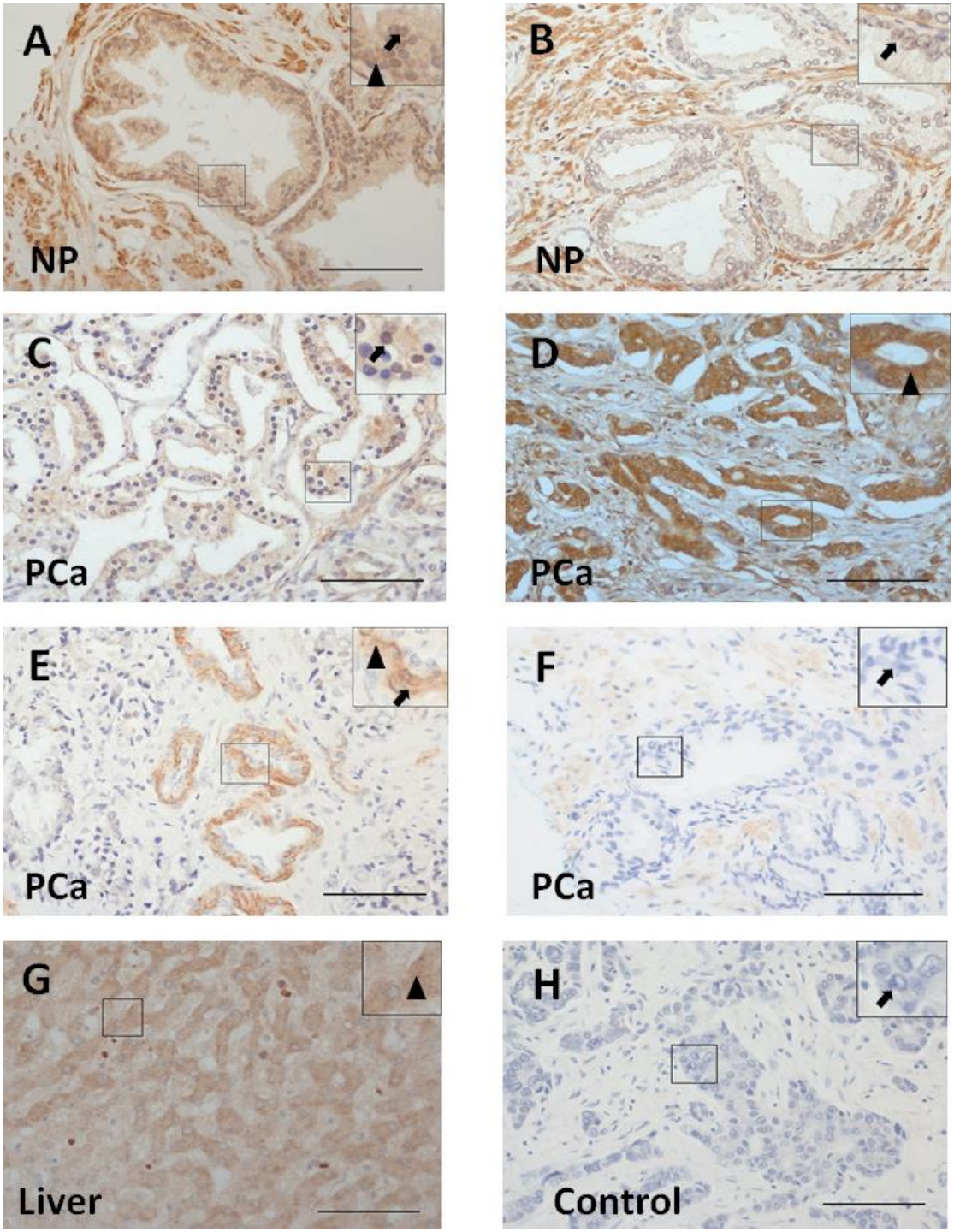

Figure 5. 2 Sox7 staining in samples from the Bath cohort. Sox7 was expressed heterogeneously in both normal and malignant tissues of the prostate, using anti-Sox7 A antibody. (A) Strong nuclear (Black arrow) and weak cytoplasmic (Black arrowhead) anti-Sox7 A staining in NP. (B) Weak nuclear (Black arrow) anti-Sox7 A staining in NP. (C) Strong nuclear (Black arrow) anti-Sox7 A staining in PCa. (D) Strong cytoplasmic anti-

Sox7 A staining (Black arrowhead) in PCa. (E) Weak nuclear (Black arrow) and cytoplasmic (Black arrowhead) anti-Sox7 A staining in PCa. (F) PCa showed no detectable staining for Anti-Sox7 A (Blackarrow). (G) Positive control: Strong cytoplasmic anti-Sox7 A staining (Black arrowhead) in normal liver tissue. (H) Negative control (no primary antibody added) showed no detectable background staining (Black arrow) in PCa. Scale bars=100µm with inserts at 2x zoom.

5.2.1.2 Association between anti-Sox7 immunostaining and histopathological parameters of prostate cancer in the Bath cohort

Having carried out IHC staining on the TMA prostate tissue samples, using anti-Sox7 A antibody, the nuclear and cytoplasmic anti-Sox7 A staining was assessed using the proportion and intensity 2 scores (Described in Chapter 2.3.2) and then compared between normal vs malignant prostate tissues, different primary Gleason grades, clinical stages and biochemical recurrence, using the clinical data available for the Bath cohort. The range of nuclear and cytoplasmic anti-Sox7 A scores varied between 0– 5.8 and 0-5.2, respectively (Figure 5.3). The potential association between anti-Sox7 A IHC results and PCa histopathological parameters was then analysed and is described below.

The statistical analysis looked first at the expression of anti-Sox7 A in normal and malignant prostate tissues. Quantification of the IHC staining showed nuclear and cytoplasmic anti-Sox7 A staining reduced significantly in PCa compared to NP tissues (p=0.0152, 0.0205 respectively) (Figure 5.3 (A&B) & Table 5.2). Nuclear and cytoplasmic anti-Sox7 A staining was also reduced significantly in PCa patients with a primary Gleason grade 4 compared to those with a primary Gleason grade 3 (p= 0.0073, 0.0405, respectively) (Figure 5.3 (C&D) & Table 5.2). In contrast, nuclear and cytoplasmic anti-Sox7 A staining was not associated significantly with PCa primary tumour stage T (T1-2 vs T3-4) (p=0.9732 & 0.4676, respectively) (Figure 5.3 E&F & Table 5.2). Finally, nuclear and cytoplasmic anti-Sox7 A staining showed a slight significant reduction in recurrent PCa patients compared to those with non-recurrent (p=0.0424 & 0.0479 respectively) (Figure 5.3 (G&H) & Table 5.2).

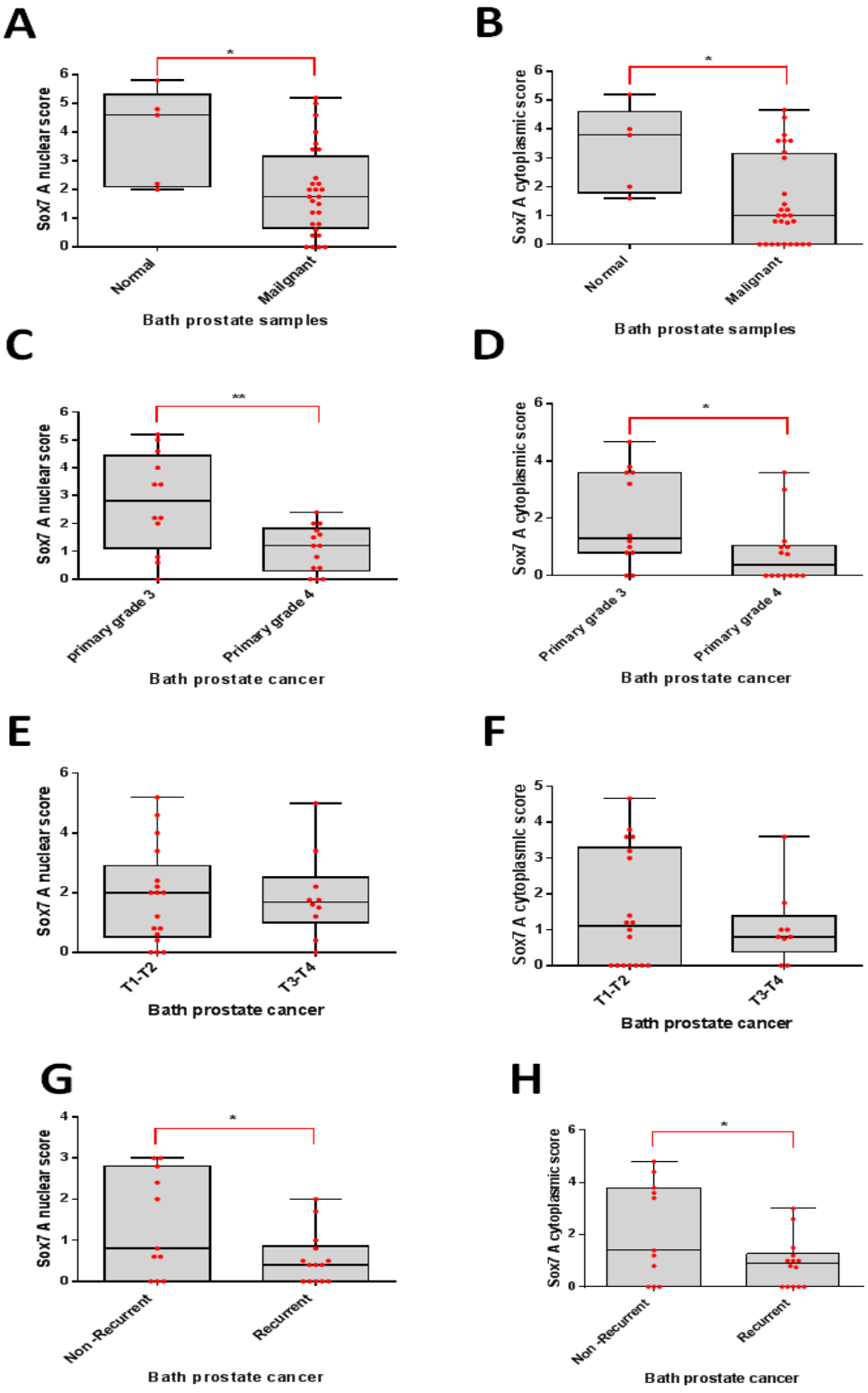

Figure 5. 3 Quantification of nuclear and cytoplasmic anti-Sox7 A staining in both normal and malignant Bath prostate tissues. Immunohistochemical staining of anti-Sox7 A was quantified using proportion and intensity 2 scores for nuclear and cytoplasmic IHC staining. (A) Nuclear anti-Sox7 A staining was significantly reduced in PCa compared to NP (p=0.0152). (B) Cytoplasmic anti-Sox7 A staining was also significantly reduced in PCa compared to NP (p=0.0205). (C) Nuclear anti-Sox7 A staining was significantly reduced in primary Gleason grade 4 compared to grade 3 (p=0.0073). (D) Cytoplasmic anti-Sox7 A staining was significantly reduced in primary Gleason grade 4 compared to grade 3 (p= 0.0405). (E) Nuclear anti-Sox7 A staining was

not associated significantly with PCa clinical stage T (p=0.9732). (F) Cytoplasmic anti-Sox7 A staining was not associated significantly with PCa clinical stage T (p=0. 4676). (G) Nuclear anti-Sox7 A staining was reduced significantly in a patient with recurrent PCa compared to those with a non-recurrent (p= 0.0424). (H) Cytoplasmic anti-Sox7 A staining was also reduced significantly in a patient with a recurrent PCa compared to those with a non- recurrent (p=0.0479). The mean of five random fields was taken per prostate sample. Statistical significance was determined with unpaired t-test for each set of conditions. NP(n=5) PCa (n=28), grade 3 (n=12), grade 4 (n=14), recurrent PCa (n=14) and non- recurrent PCa (n=11).

Table 5. 2 Nuclear and cytoplasmic anti-Sox7 A staining results with clinical data of the Bath cohort

Comparison	Nuclear anti- Sox7 A staining		Cytoplasmic anti-Sox7 A staining	
	Results	p. value	Results	p. value
Normal vs malignant	Lower in malignant	0.0152	Lower in malignant	0.0205
Primary Gleason grades (3 & 4)	Lower in high grade	0.0073	Lower in high grade	0.0405
Clinical Stage (T)	No statistically significant difference	0.9732	No statistically significant difference	0.4676
5 years Biochemical recurrence	Slightly lower in recurrent	0.0424	Slightly lower in recurrent	0.0479

In summary, nuclear and cytoplasmic anti-Sox7 A staining was reduced significantly in PCa compared to NP tissues and was also negatively associated with increasing primary Gleason grade and biochemical recurrence, but not with the clinical stage (T). These results confirmed our hypothesis about Sox7 expression in PCa and its association with histopathological parameters, including primary Gleason grade and biochemical relapse. However, the results should be treated as preliminary data because of the small sample size, so the staining was repeated using the tissue microarray with a larger sample size.

5.2.3 Expression of Sox7 in the TMA cohort, using anti-Sox7 A antibody

5.2.3.1 Immunohistochemical expression of anti-Sox7 A in the normal and malignant TMA prostate samples.

IHC staining of samples from the TMA showed nuclear anti-Sox7 A staining in the glandular regions of all NP tissues (16/16, 100%) with variable levels of staining, ranging from strong (Figure 5.4 A, arrow) to weak staining (Figure 5.4 B, arrow). Nuclear anti-Sox7 A staining was also observed in PCa tissues and the intensity of signal varied widely, ranging from a strong and widespread (Figure 5.4 C&D, arrows), weak and scattered (Figure 5.4 E, arrow) or negative (Figure 5.4 F).

In addition to the nuclear staining, the TMA prostate tissue samples had cytoplasmic anti-Sox7 A staining, with different levels of staining pattern between cases, ranging from strong (Figure 5.4 D arrowheads), moderate (Figure 5.4 A, arrowhead), weak (Figure 5.4 B & F, arrowheads), and negative (Figure 5.4 G). A negative control (no primary antibody) showed no detectable background staining (Figure 5.4 H, arrow). The IHC of the TMA prostate sample result was consistent with the Bath cohort results.

5.2.3.2 Association between anti-Sox7 A immunostaining and histopathological parameters of prostate cancer in the TMA cohort

Having carried out IHC staining, the nuclear and cytoplasmic anti-Sox7 A staining was then quantified using the proportion and intensity 2 score, as carried out for the Bath cohort samples. The range of nuclear and cytoplasmic anti-Sox7 A scores varied between 0-6 (Figure 5.5). The potential association between anti-Sox7 A IHC results and PCa histopathological parameters was then analysed and is described below (Figure 5.5 and Table 5.3).

The statistical analysis looked at the expression of anti-Sox7 A in cancerous and non-cancerous prostate tissue samples. Quantification of the IHC staining showed a significant reduction of nuclear and cytoplasmic anti-Sox7 A staining in PCa compared to NP tissues ($p = < 0.0001$) (Figure 5.5 (A&B) & Table 5.3). Nuclear and cytoplasmic Anti-Sox7 A staining showed a significant difference among different primary Gleason grade groups ($p = 0.0084$ & 0.0488, respectively) (Figure 5.5 (C&D), Table 5.3). The multi-comparison Tukey's tests showed reduced nuclear and cytoplasmic anti-Sox7 A staining significantly within increasing primary Gleason grade. This significant reduction was only shown between nuclear and cytoplasmic anti-Sox7 A when comparing PCa patients with a primary grade 5 to those with a grade 3 ($p = 0.0061$ & 0.0429, respectively) (Figure 5.5 (C&D) &Table 5.3). In contrast, nuclear and cytoplasmic anti-Sox7 A staining showed no significant difference when comparing PCa patients with a primary Gleason grade 4 to those with a grade 3 ($p = 0.1454$ & 0.1046, respectively) or a grade 5 ($p = 0.1247$&0.67, respectively) (Figure 5.5 (C&D) &Table 5.3). Nuclear and cytoplasmic anti-Sox7 A staining data was then examined in samples from different clinical stages, including primary tumour stages (T1-2 vs T3-4), regional lymph node (N0 vs N1) and distant metastasis (M0 vs M1), using the clinical data available for the TMA cohort. There was no statistical difference between nuclear and cytoplasmic anti-Sox7 A staining and PCa stage (Table 5.3).

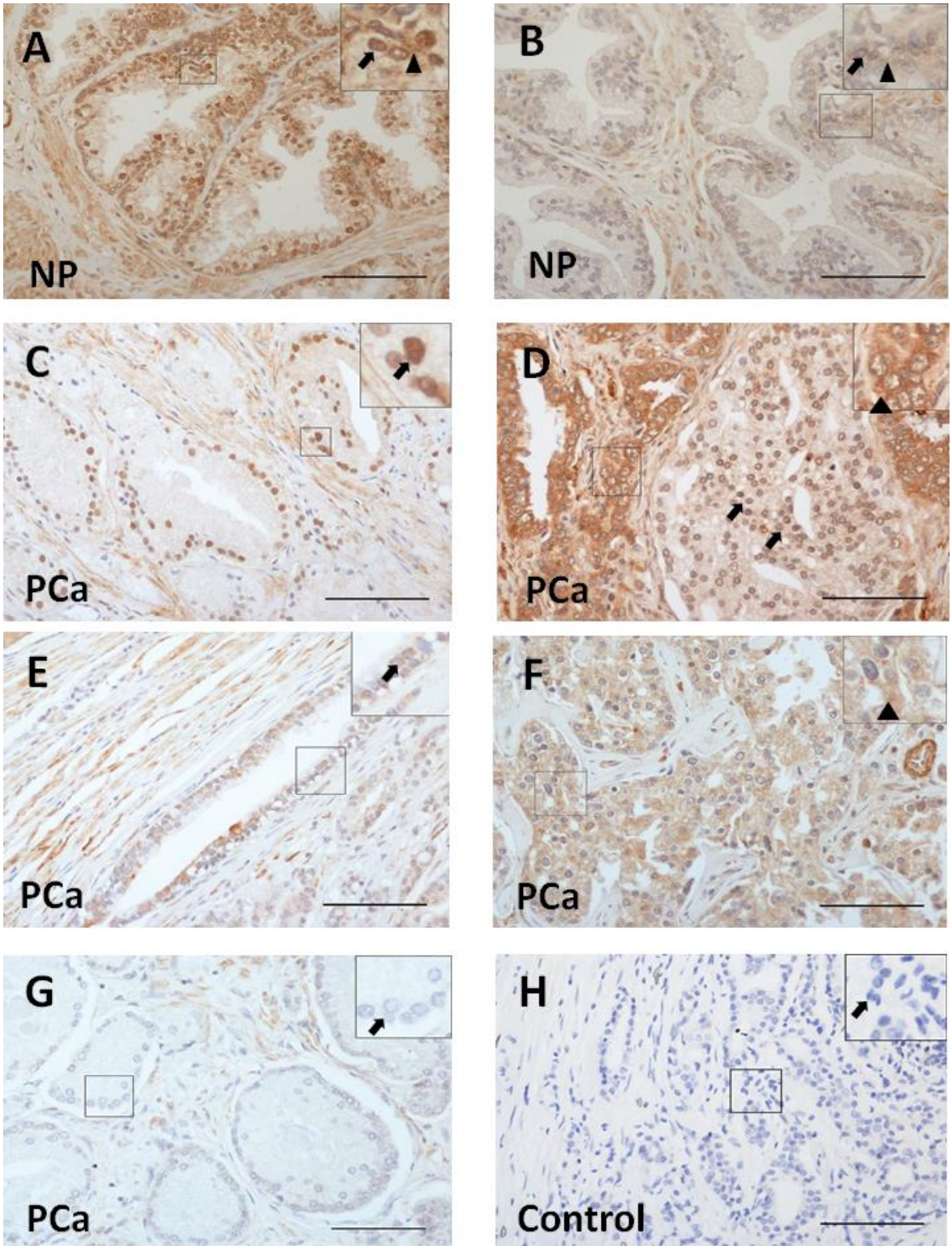

Figure 5. 4 Anti-Sox7 staining in samples from the TMA cohort. Anti-Sox7 A was expressed heterogeneously in both normal and malignant tissues of the prostate. (A) Strong nuclear (Black arrow) and moderate cytoplasmic (Black arrowhead) anti-Sox7 A staining in NP. (B) Weak nuclear (Black arrow) and cytoplasmic (Black arrowhead) anti-Sox7 staining in NP. (C) Strong nuclear (Black arrow) anti-Sox7 A staining in PCa. (D) Strong nuclear (Black arrow) and cytoplasmic (Black arrowhead) anti-Sox7 A staining in PCa. (E) Weak nuclear (Black arrowhead) anti-Sox7 A staining in PCa. (F) Weak cytoplasmic (Black arrowhead) anti-Sox7 A staining in PCa. (G) Negative anti-Sox7 A staining (Black arrow) in PCa. (H) Negative control (no primary antibody added) shows no background staining (Black arrow) in PCa. Scale bars=100µm with inserts at 2x zoom.

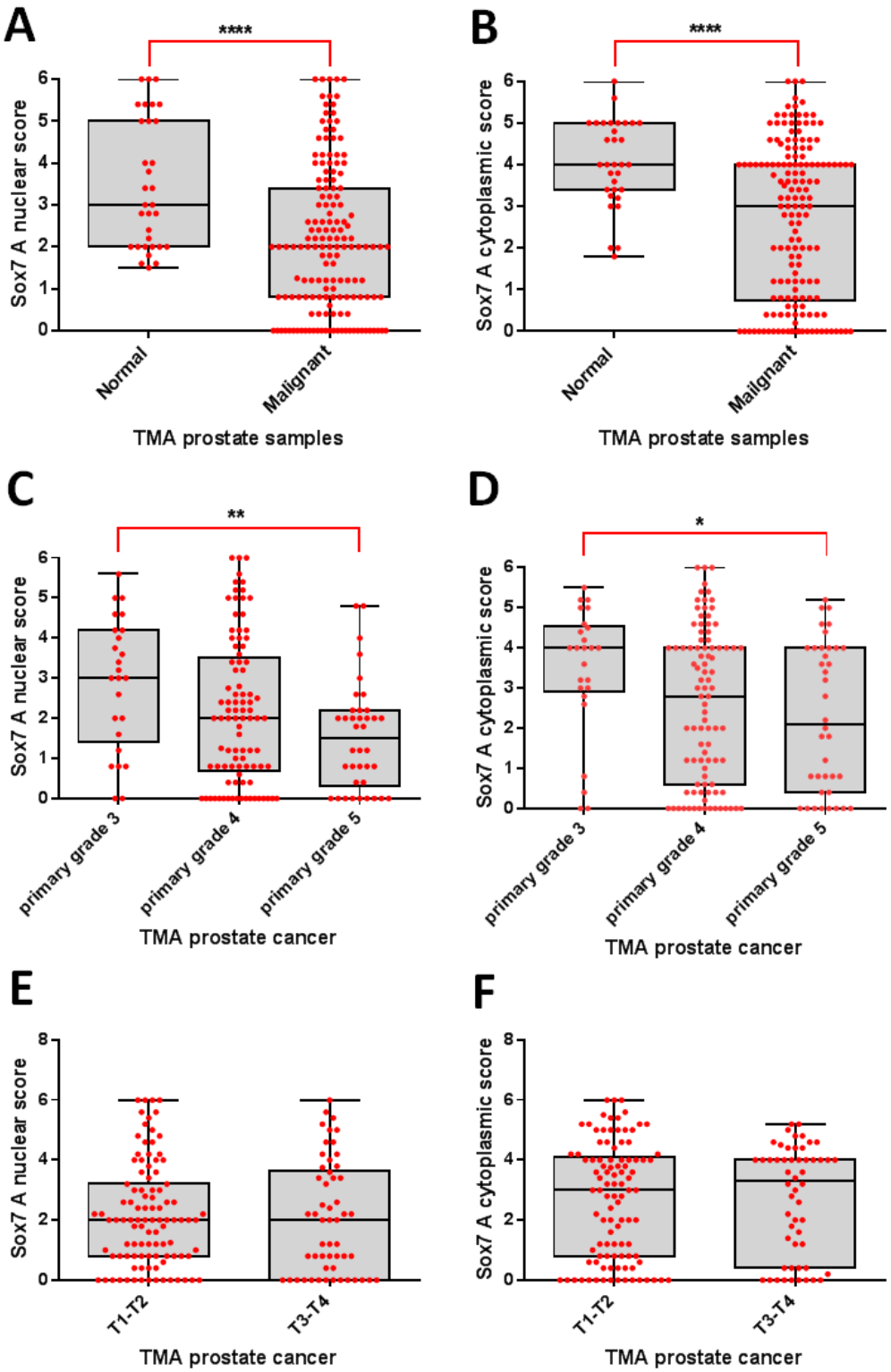

Figure 5. 5 Quantification of nuclear and cytoplasmic anti-Sox7 A staining in both normal and malignant TMA prostate tissues, Immunohistochemical staining of anti-Sox7 A was quantified in TMA group using the proportion and intensity 2 scores for nuclear and cytoplasmic IHC staining. (A) Nuclear anti-Sox7 A staining was significantly reduced in PCa compared to NP tissues (p= < 0.0001). (B) A significant reduction of cytoplasmic anti-Sox7 A staining was in PCa compared with NP (P= < 0.0001). (C) Nuclear anti-Sox7 A staining showed a significant difference among different primary Gleason grade (p= 0.0084) and multiple comparison tests (Tukey) showed a negative association with increasing primary Gleason grades. This reduction was only statistically significant between primary Gleason grade 5 & 3 (p= 0.0061). (D) Cytoplasmic anti-Sox7 A staining showed a significant difference among different primary Gleason scores (P= 0.0088). Cytoplasmic anti-SOX7 staining was negatively associated with increasing grade, using multiple comparison

tests (Tukey). This reduction of cytoplasmic anti-Sox7 A was observed only between primary Gleason grade 5 and 3 (p= 0.0429). Data represent the mean of five random images per case. Unpaired or one-way ANOVA tests were conducted to determine the statistical difference for each set of conditions. NP (n=16), PCa (n=80), grade 3 (n=13), grade 4 (n=46) and grade 5 (n=18).

Table 5. 3 Summary of nuclear and cytoplasmic anti-Sox7 A staining results with clinical data in the TMA cohort.

Comparison	**Nuclear anti-Sox7 A staining**			**Cytoplasmic anti-Sox7 A staining**		
	Results		**p. value**	**Results**		**p. value**
Normal vs malignant	Lower in malignant		< 0.0001	Lower in malignant		<0.0001
Primary Gleason Grade (3,4 &5)	Lower in high Gleason grade	Anova test	0.0084	Lower in high Gleason grade	Anova test	0.0488
		Grade 4 vs. Grade 3	0.1454		Grade 4 vs. Grade 3	0.1048
		Grade 5 vs. Grade 3	0.0061		Grade 5 vs. Grade 3	0.0429
		Grade 5 vs. Grade 4	0.1247		Grade 5 vs. Grade 4	0.67
Stage (T)	No statistically significant difference		0.685	No statistically significant difference		0.9196
Stage (M)	No statistically significant difference		0.9364	No statistically significant difference		0.1572
Stage (N)	No statistically significant difference		0.5601	No statistically significant difference		0.5595

In summary, nuclear and cytoplasmic anti-Sox7 A staining was reduced significantly in PCa and was negatively associated with increasing Gleason grades, but not with stage (TNM). This result largely agreed with the Bath cohort results.

5.2.4 Expression of Sox7 in the Bath cohort using the anti-Sox7 B antibody

In order to increase confidence in the results showing a reduction in Sox7 expression, it was important to confirm them using a second antibody, independent from the anti- Sox7 rabbit polyclonal antibody from Sigma. This helps confirm that the staining is specific for Sox7 and not cross-reactive with other proteins. A second antibody was identified which was raised against a different immunogenic part of Sox7 (Figure 5.6) and is referred to here as the anti-Sox7 B antibody.

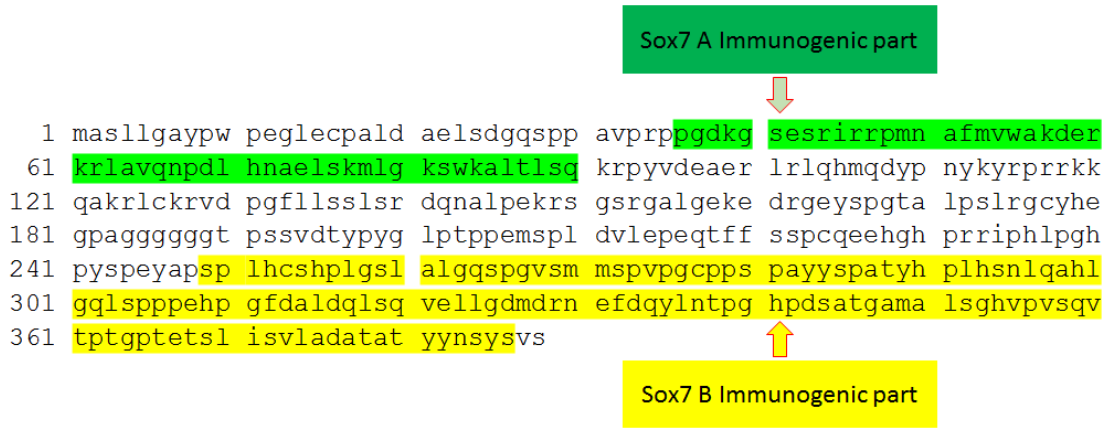

Figure 5. 6 Sox7 protein sequence with the immunogenic part of the two different antibodies highlighted. Sox7 protein consists of 388 amino acids and the immunogen for each antibody was in a different region of the protein. The immunogenic part of the anti-Sox7 A antibody was in the N terminal amino acids 37-86 (Green highlighted). The anti-Sox7 B antibody was raised against an immunogen at the C terminal of Sox7 (Yellow highlighted).

5.2.4.1 Immunohistochemical staining patterns of the two Sox7 antibodies

In order to confirm that the two independent Sox7 antibodies were producing a similar staining pattern, IHC was carried out using sections from similar regions of prostate and liver tissue samples from the Bath cohort. The IHC showed that both Sox7 antibodies had a very similar staining pattern in prostate and liver tissues (Figure 5.7). In prostate tissue, anti-Sox7 A& B staining was found in the glandular (Figure 5.7 A&B arrows) and stromal regions (Figure 5.7 A&B arrowheads). A moderate nuclear Sox7 staining was also observed in prostate tissues, using anti-Sox7 A & B (Figure 5.7 C&D arrows, respectively). In addition to its expression in prostate tissue, cytoplasmic Sox7 staining was also observed in normal liver tissue, using the two different antibodies (Figure 5.7 E & F arrowheads).

The two different Sox7 antibodies had the same staining pattern in prostate and liver tissues samples so it was decided to carry out IHC staining of the prostate samples from the Bath and TMA cohorts using the anti-Sox7 B antibody.

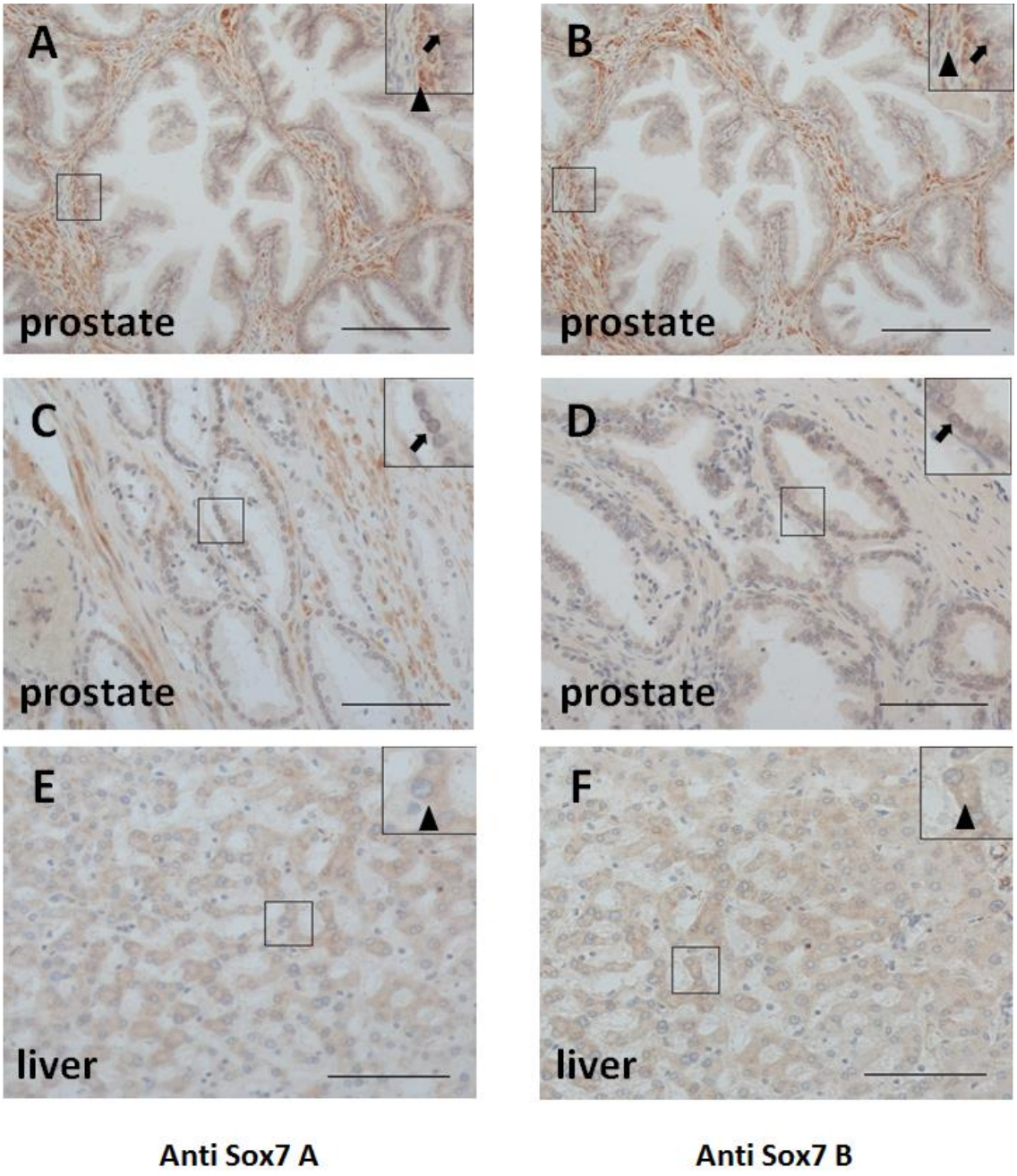

Figure 5. 7 Two distinct Sox7 antibodies show the expected patterns of Sox7 expression in the Bath prostate and liver tissue samples. (A) Moderate glandular (Black arrow) and strong stromal (Black arrowhead) anti-Sox7 A staining in prostate tissue. (B) Moderate glandular (Black arrow) and strong stromal (Black arrowhead) anti-Sox7 B staining in prostate tissue. (C) Moderate nuclear anti-Sox7 A staining in prostate tissues. (D) Moderate nuclear anti-Sox7 B staining in prostate tissues. (E) Weak cytoplasmic staining (Black arrowhead) anti-Sox7 A staining in liver tissue. (F) Weak cytoplasmic staining (Black arrowhead) anti-Sox7 B staining in liver tissue. Both Sox7 antibodies showed very similar staining patterns in prostate and liver tissues. Scale bars—100 μm with inserts at 2x zoom.

5.2.5 Expression of Sox7 in the Bath cohort, using anti-Sox7 B antibody

5.2.5.1 Anti-Sox7 B staining in the normal and malignant Bath prostate samples

IHC was carried out on normal and malignant prostate tissues from the Bath cohort, using anti-Sox7 B antibody. The IHC staining showed nuclear anti-Sox7 B staining in NP tissues and ranged from strong (Figure 5.8 A, arrow) to weak (Figure 5.8 B, arrow). Nuclear anti-Sox7 B staining was also detected in PCa tissues and the intensity of signal varied widely, from strong (Figure 5.8 C, arrow), moderate (Figure 5.8 D, arrow), weak (Figure 5.8 E, arrow) and negative (Figure 5.8 F, arrow).

In addition to the nuclear staining, Sox7 was also expressed in the cytoplasm of the glandular epithelial cells of both normal and malignant prostate tissues from the Bath cohort, using anti-Sox7 B antibody. The staining patterns of anti-Sox7 B varied between cases, ranging from strong (Figure 5.8 A& C, arrowheads), moderate (Figure 5.8 D, arrowhead), weak (Figure 5.8 E, arrowhead) and negative (Figure 5.8 F, arrow). The IHC data showed cytoplasmic anti- Sox7 B staining in the positive control liver tissues (Figure 5.8 G, arrowhead). A negative control showed no significant background staining in prostate tissue (Figure 5.8 H, arrow). This result appeared very similar to that obtained using the anti-Sox7A antibody.

5.2.5.2 Association between anti-Sox7 B immunostaining and histopathological parameters of prostate cancer in the Bath cohort

Having carried out IHC staining, the nuclear and cytoplasmic anti-Sox7 B staining was then quantified using the proportion and intensity 2 scores, respectively (Described in Chapter 2.3.2) and then compared between normal vs. malignant prostate tissues, primary Gleason grades, clinical stages. The range of nuclear and cytoplasmic anti-Sox7 B scores varied between 0– 5.8 (Figure 5.9). Since this analysis was carried out later in the project and the number of samples still available from the Bath cohort was reduced, for example, 3 NP vs. 17 PCa, statistical tests were not carried out.

Quantification of the IHC showed that PCa tissues had lower nuclear and cytoplasmic anti-Sox7 B scores compared to NP tissues (Figure 5.9 (A&B) & Table 5.4). Nuclear and

cytoplasmic anti-Sox7 B staining was also reduced in PCa tissues with a primary Gleason grade 4 compared to those with a grade 3 (Figure 5.9 (C&D) & Table 5.4).

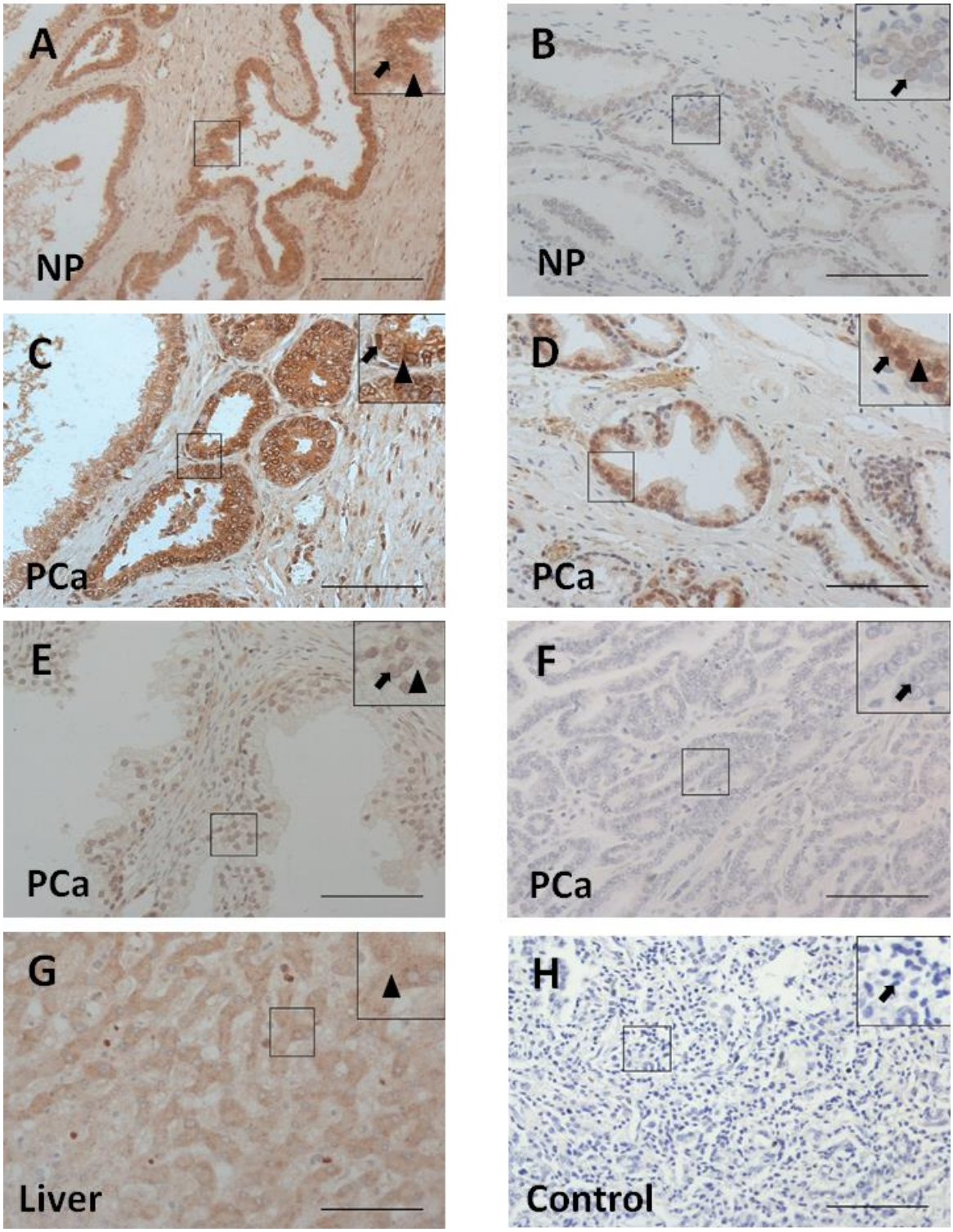

Figure 5. 8 Sox7 staining in samples from the Bath cohort. Sox7 was expressed heterogeneously in both normal and malignant tissues of the prostate, using anti-Sox7 B antibody. (A) Strong nuclear (Black arrow) and cytoplasmic (Black arrowhead) anti-Sox7 B staining in NP. (B) Weak nuclear (Black arrow) anti-Sox7 B staining in NP. (C) Strong nuclear (Black arrow) and cytoplasmic (Black arrowhead) anti-Sox7 B staining in PCa. (D) Moderate nuclear (Black arrow) and cytoplasmic anti-Sox7 B staining (Black arrowhead) in PCa. (E) Weak nuclear (Black arrow) and cytoplasmic (Black arrowhead) anti- Sox7 B staining in PCa. (F) PCa tissue had negative staining for anti-Sox7 B (Black arrow). (G) Positive control: Cytoplasmic anti-Sox7 B staining

(Black arrowhead) in normal liver tissue. (H) Negative control (no primary antibody added) showed no background staining (Black arrow) in PCa. Scale bars=100µm with inserts at 2x zoom.

Nuclear and cytoplasmic Sox7 staining trended to be lower in PCa patients with clinical stage T3-4 compared to those with a T1-2 (Figure 5.9 (E&F) & Table 5.4). In contrast, IHC quantification showed nuclear and cytoplasmic anti-Sox7 B scores trended to be lower in recurrence compared to non-recurrence (Figure 5.9 (G&H) & Table 5.4). This result largely agreed with anti-Sox7 A IHC results in the Bath cohorts.

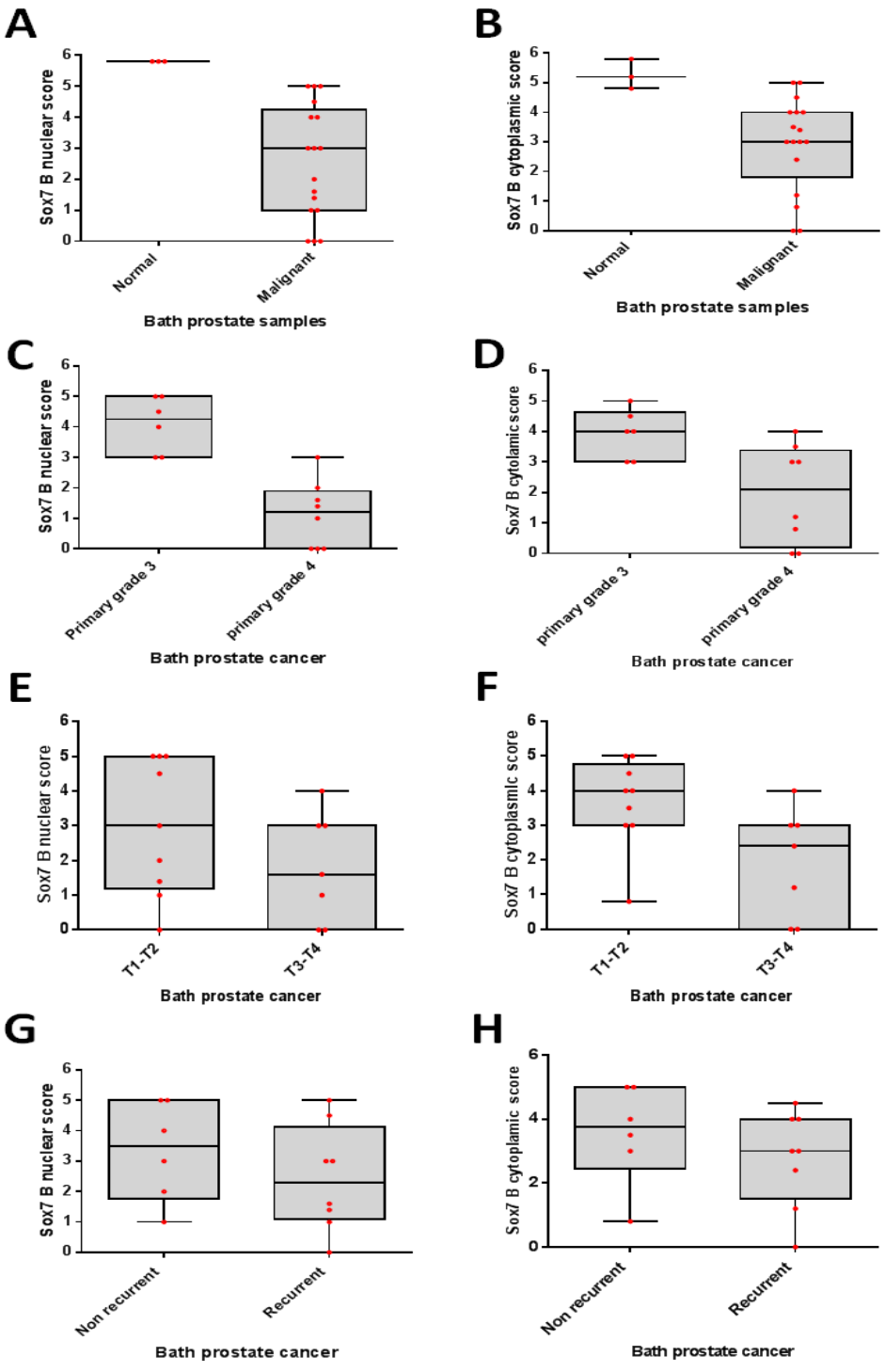

Figure 5. 9 Quantification of nuclear and cytoplasmic anti-Sox7 B staining in both normal and malignant Bath prostate tissues, using anti-Sox7 B antibody. IHC staining of anti-Sox7 B was quantified in the Bath cohort the proportion and intensity 2 scores for nuclear and cytoplasmic IHC staining. (A) Nuclear anti-Sox7 B staining was reduced in PCa compared to NP. (B) Cytoplasmic anti-Sox7 B staining was reduced in PCa compared to NP. (C) Nuclear anti-Sox7 B staining was reduced in Gleason grade 4 compared to Gleason grade 3. (D) Cytoplasmic anti-Sox7 B staining was reduced in Gleason grade 4 compared to Gleason grade 3. (E) Nuclear anti-Sox7 B staining trended to be lower in advanced stage T3-4 (F) Cytoplasmic anti-Sox7 B staining trended to be lower in advanced stage T3-4. (G) Nuclear anti-Sox7 B staining trended to be lower recurrent PCa compared to a non-recurrent. (H) Cytoplasmic anti-Sox7 B staining trended to be lower recurrent PCa compared to a non-recurrent. NP (n=3) PCa (n=17), grade 3 (n=6), grade 4 (8), T1-T2 (n= 9), T3-T4 (n= 7), non-recurrent (n= 6) and recurrent (n=8).

Table 5. 4 Summary of nuclear and cytoplasmic Sox7 B staining results with clinical data in the Bath cohort. The small sample size meant that statistical analysis was not carried out.

Comparison	**Nuclear anti- Sox7 B staining**	**Cytoplasmic anti-Sox7 B staining**
	Results	**Results**
Normal vs malignant	Lower in malignant	Lower in malignant
Primary Gleason grades (3 &4)	Lower in high grade	Lower in high grade
Clinical Stage (T)	Trend to be lower in T3-4	Trend to be lower in T3-4
5 years Biochemical recurrence	Trend to be lower in recurrence	Trend to be lower in recurrence

In summary, nuclear and cytoplasmic anti-Sox7 B staining was reduced in PCa compared to NP tissues and was negatively associated with increasing Gleason grade, clinical stage and biochemical relapse. The results were agreed with anti-Sox7 A results in both cohorts. However, the staining should be treated as preliminary data because of the small sample size, so the staining was repeated using a tissue microarray with a larger sample size.

5.2.6 Expression of Sox7 in the TMA cohort, using anti-Sox7 B antibody

5.2.6.1 Immunohistochemical expression of anti-Sox7 B in the normal and malignant TMA prostate samples

Expression of Sox7 was investigated by IHC on the TMA prostate tissue samples, using anti-Sox7 B antibody. IHC results showed that NP tissues had nuclear anti-Sox7 B staining that ranged from strong (Figure 5.10, A, arrow) to weak (Figure 5.10 B, arrow). Nuclear anti-Sox7 B staining was also observed in PCa tissues and the intensity of signal varied widely, from strong (Figure 5.10 C, arrow), moderate (Figure 5.10 D&E, arrows) and weak (Figure 5.10 F&G, arrows).

In addition to the nuclear staining, both normal and malignant prostate tissues showed cytoplasmic anti-Sox7 B staining with variable staining levels, ranging from moderate (Figure 5.10 C, arrowhead), weak (Figure 5.10 B, D &F, arrowheads) and negative (Figure

5.10 F). A negative control showed no significant background staining in prostate tissue (Figure 5.10 H, arrow). This result largely agreed with anti-sox7 A results in both cohorts.

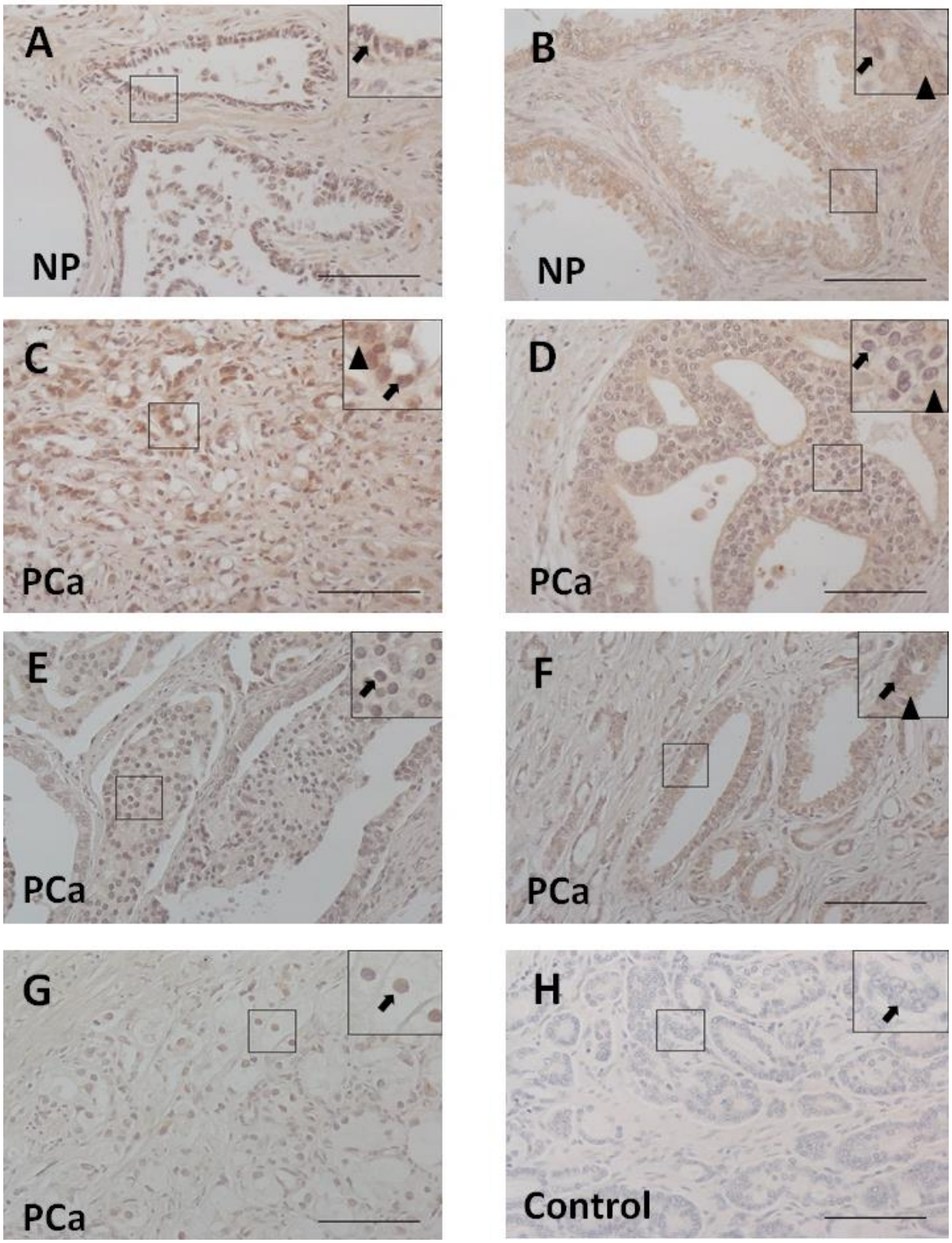

Figure 5.10 Anti-Sox7 B staining in samples from the TMA cohort. Anti-Sox7 B was expressed heterogeneously in both normal and malignant tissues of the prostate. (A) Strong nuclear (Black arrow) anti-Sox7 B staining in NP. (B) Weak nuclear (Black arrow) and cytoplasmic (Black arrowhead) anti-Sox7 B staining in NP. (C) Strong nuclear (Black arrow) and moderate cytoplasmic (Black arrowhead) anti-Sox7 B staining in PCa. (D) Moderate nuclear (Black arrow) and weak cytoplasmic (Black arrowhead) anti-Sox7 B staining in PCa. (E) Moderate nuclear (Black arrow) anti-Sox7 B staining in PCa. (F) Weak nuclear (Black arrow) and cytoplasmic (Black arrowhead) anti-Sox7 B staining in PCa. (G) Weak nuclear (Black arrow) anti-

Sox7 B staining in PCa. (H) Negative control (no primary antibody added) showed no detectable background staining (Black arrow) in PCa. Scale bars=100µm with inserts at 2x zoom.

5.2.6.2 Association between anti-Sox7 B immunostaining and histopathological parameters of prostate cancer in the TMA cohort

Having carried out IHC staining, the nuclear and cytoplasmic anti-Sox7 B staining was then quantified using the proportion and intensity 2 scores (Described in Chapter 2.3.2). The range of nuclear and cytoplasmic anti-Sox7 B scores varied between 0 – 6 (Figure 5.11). The potential association between anti-Sox7 B IHC results and histopathological parameters of PCa was then analysed and is described below.

The analysis looked first at the expression of Sox7 in cancerous vs. non-cancerous prostate tissue, using anti-Sox7 B antibody. Quantification of the IHC showed reduced nuclear and cytoplasmic anti-Sox7 B staining significantly in PCa compared to NP tissues (p= < 0.0001) (Figure 5.11 (A&B) & Table 5.5). Nuclear and cytoplasmic anti-Sox7 B staining showed a significant difference among different Gleason grade groups (p= < 0.0001, respectively) (Figure 5.11 (C&D)& Table 5.5). Detailed analysis, using multi-comparison (Tukey) tests showed that nuclear and cytoplasmic anti-Sox7 B staining was negatively associated with increasing primary Gleason grades. Nuclear and cytoplasmic anti-Sox7 A staining was significantly reduced when comparing PCa patients with a grade 5 to those with a grade 3 (p= < 0.0001) or a grade 4 (p= < 0.0001) (Figure 5.9 (C&D)& Table 5.4). Cytoplasmic anti-Sox7 B staining was also reduced significantly in a Gleason grade 4 compared to Gleason grade 3 (p=<0.0001) (Figure 5.11 D & Table 5.5).

There was no statistical association between nuclear anti-Sox7 B staining and PCa stage (Table 5.5). In contrast, cytoplasmic anti-Sox7 B staining was negatively associated with clinical stage T (P= 0.0085) (Figure 5.11 F & Table 5.5), but not associated with other clinical stages, including N (N0 vs N1) (P= 0.0098) and M (M0 vs M1) (P= 0.136) (Table 5.5).

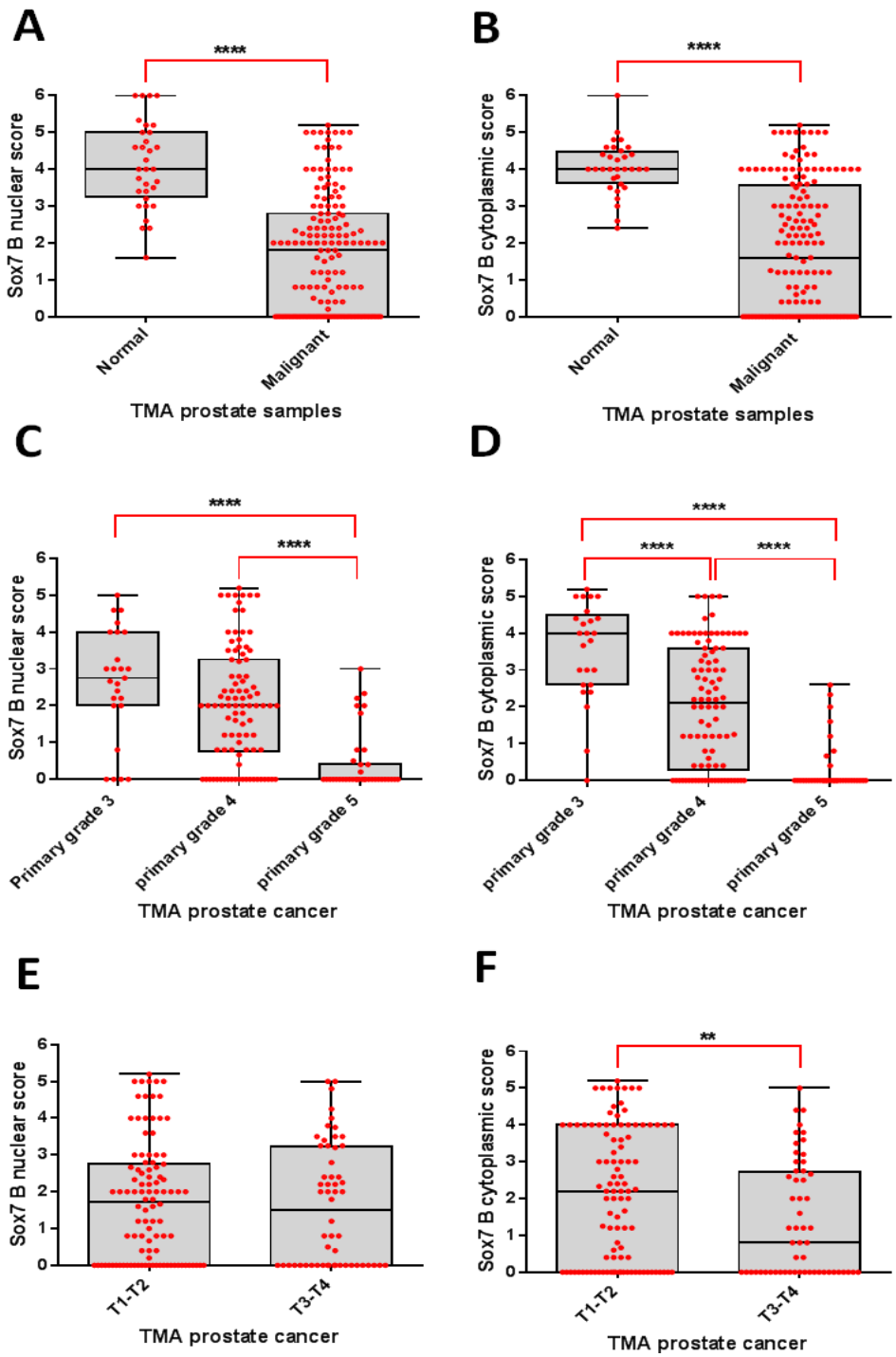

Figure 5. 11 Quantification of nuclear and cytoplasmic Sox7 staining in both normal and malignant TMA prostate tissues, using anti-Sox7 antibody. Immunohistochemical staining of anti-Sox7 B was quantified in the TMA group using the proportion and intensity 2 scores for nuclear and cytoplasmic IHC staining. (A) Nuclear anti-Sox7 B staining was significantly decreased in PCa compared to NP tissues (p= < 0.0001). (B) A significant reduction of cytoplasmic anti-Sox7 B staining was in PCa compared to NP tissues (p= < 0.0001). (C) Nuclear anti-Sox7 B staining showed a significant difference among different Gleason grades (p= <0.0001) and multiple comparison tests (Tukey) showed a significant reducing with increasing primary Gleason grades. This reduction was statistically significant when comparing PCa patient with a Gleason grade 5 to grade 3 (p= <0.0001) or grade 4 (p=<0.0001) only. (D) Cytoplasmic anti-Sox7 B staining showed a significant difference among different primary Gleason grades (p= <0.0001). Multiple comparison tests (Tukey) showed that this reduction was statistically significant when comparing PCa patient with a primary Gleason grade 5 to grade 3 (p= <0.0001) or grade 4 (p=<0.0001) and when comparing grade 4 tissues to those with grade 3 (p=<0.0001). (E) Nuclear anti-Sox7 B staining was not associated with primary tumour volume (p=0.6656). (F) Cytoplasmic

anti-Sox7 B was negatively associated with clinical stage T (p= 0.0085). Unpaired or one-way ANOVA tests were conducted to determine the statistical difference for each set of conditions. PCa (n=80) and NP (n=16), grade 3 (n=13), grade 4 (n=46) and grade 5 (n=18), T1-2 (n= 51) and T3-4 (n= 28).

Table 5. 5 Summary of nuclear and cytoplasmic anti-Sox7 B staining results with the TMA clinical data

Comparison	**Nuclear anti-Sox7 B staining**			**Cytoplasmic anti-Sox7 B staining**		
	Results		**p. value**	**Results**		**p. value**
Normal vs malignant	Lower in malignant		< 0.0001	Lower in malignant		<0.0001
Primary Gleason Grade (3,4 &5)	Lower in high Gleason grade	Anova test	< 0.0001	Lower in high Gleason grade	Anova test	< 0.0001
		Grade 4 vs. Grade 3	0.1541		Grade 4 vs. Grade 3	< 0.0001
		Grade 5 vs. Grade 3	< 0.0001		Grade 5 vs. Grade 3	< 0.0001
		Grade 5 vs. Grade 4	< 0.0001		Grade 5 vs. Grade 4	< 0.0001
Stage (T)	No statistically significant difference		0.6656	Lower in T3-4		0.0085
Stage (M)	No statistically significant difference		0.3205	No statistically significant difference		0.136
Stage (N)	No statistically significant difference		0.1808	No statistically significant difference		0.0988

In summary, nuclear and cytoplasmic anti-Sox7 B staining was reduced significantly in PCa compared to NP tissues and was negatively associated with increasing primary Gleason grades. There was no association with stage, except for the cytoplasmic anti-Sox7 B staining which was negatively associated with clinical stage T. This result largely agreed with the Bath cohort and the anti-Sox7 A result in both cohorts.

2.5.7 Sox7 immunostaining in the Bath and TMA cohorts: testing the hypothesis

The results from the two cohorts and if the hypothesis is accepted or rejected is summarised in Table 5.6.

Table 5. 6 The summary of Sox7 results in the Bath and TMA cohorts

Parameters	Localisation	Hypothesis	Bath	TMA	Results Confirmed?	Hypothesis Accepted?
Normal vs Cancer	Nuclear Sox7 A	Sox7 will be reduced in PCa	Sox7 is reduced in PCa	Sox7 is reduced in PCa	Yes	Yes
	Cytoplasmic Sox7 A		Sox7 is reduced in PCa	Sox7 is reduced in PCa	Yes	Yes
	Nuclear Sox7 B		Sox7 is reduced in PCa	Sox7 is reduced in PCa	Yes	Yes
	Cytoplasmic Sox7 B		Sox7 is reduced in PCa	Sox7 is reduced in PCa	Yes	Yes
Primary Gleason grades	Nuclear Sox7 A	Sox7 will be reduced in high grade	Sox7 is reduced in high grade	Sox7 is reduced in high grade	Yes	Yes
	Cytoplasmic Sox7 A		Sox7 is reduced in high grade	Sox7 is reduced in high grade	Yes	Yes
	Nuclear Sox7 B		Sox7 is reduced in high grade	Sox7 is reduced in high grade	Yes	Yes
	Cytoplasmic Sox7 B		Sox7 is reduced in high grade	Sox7 is reduced in high grade	Yes	Yes
Stage T	Nuclear Sox7 A	Sox7 will be reduced in advanced stage	No significant difference	No significant difference	Yes	NO
	Cytoplasmic Sox7 A		No significant	No significant difference	Yes	NO

			difference			
	Nuclear Sox7 B		No significant difference	No significant difference	Yes	NO
	Cytoplasmic Sox7 B		No significant difference	No significant difference	Yes	NO
Stage M	Nuclear Sox7 A		Not tested	No significant difference	N/A	NO
	Cytoplasmic Sox7 A		Not tested	No significant difference	N/A	NO
	Nuclear Sox7 B		Not tested	No significant difference	N/A	NO
	Cytoplasmic Sox7 B		Not tested	No significant difference	N/A	NO
Stage N	Nuclear Sox7 A		Not tested	No significant difference	N/A	NO
	Cytoplasmic Sox7 A		Not tested	No significant difference	N/A	NO
	Nuclear Sox7 B		Not tested	No significant difference	N/A	NO
	Cytoplasmic Sox7 B		Not tested	No significant difference	N/A	NO
Relapsed vs	Nuclear Sox7 A		Sox7 is reduced in	Not tested	N/A	Yes

Non-relapsed		Sox7 will be reduced in relapsed PCa	relapsed PCa			
	Cytoplasmic Sox7 A		Sox7 is reduced in relapsed PCa	Not tested	N/A	Yes
	Nuclear Sox7 B		Trend to be lower	Not tested	N/A	Yes
	Cytoplasmic Sox7 B		Trend to be lower	Not tested	N/A	Yes

5.3 Discussion

5.3.1 Summary

This study used IHC to investigate the expression of Sox7 in normal and malignant prostate tissues from the Bath and TMA cohorts and to determine if there was an association between Sox7 immunostaining and PCa clinical and histopathological parameters, including primary Gleason grade, clinical stage and biochemical relapse, using two different anti-Sox7 antibodies. The staining patterns of anti-Sox7 A& B were very similar and data from both antibodies showed that nuclear and cytoplasmic Sox7 staining was significantly reduced in PCa and was also negatively associated with increasing primary Gleason grade and risk of biochemical relapse. Sox7 staining did not show a clear association with other clinical parameters.

5.3.2 Sox7 is reduced significantly in prostate cancer and negatively associated with increasing grade and relapse

The IHC data in this study found reduced nuclear and cytoplasmic Sox7 staining significantly in PCa tissues compared to NP tissues from the two independent cohorts, using two different antibodies. This result largely agreed with previous findings for breast, liver, pancreatic, ovarian, gastric and prostate tumours (Guo *et al.*, 2008; Zhong *et al.*, 2012; Liu *et al.*, 2014; Liu *et al.*, 2016; Wang *et al.*, 2017). In particular, the PCa study in China by Zhong, *et al.* reported downregulation of Sox7 immunostaining in 147 PCa tissues compared to 28 NP tissues (Zhong *et al.*, 2012). Combining the Zhong *et al* data with the results presented in this thesis means that Sox7 has been shown to be downregulated in three

independent cohorts of PCa patients, suggesting its loss is a widespread phenomenon and not restricted to one particular patient cohort.

In the present study, Sox7 staining was negatively associated with increasing primary Gleason grade in both cohorts, using two different antibodies. This significant association has been previously reported for pancreatic and gastric tumours (Liu *et al.*, 2016; Wang *et al.*, 2017). In PCa, to our knowledge, there is only one study that has investigated the association between Sox7 expression and Gleason score and reported that there was no a significant association between them (Zhong *et al.*, 2012). The cause of this difference is not known, but might be explained as the previous prostate study in China used a different antibody. It might also be that the difference is caused by differences in the patient populations.

Our data showed that cytoplasmic anti-Sox7 B staining was reduced significantly in T3-4 compared to T1-2. However, overall, there was no clear biological association between Sox7 immunostaining and PCa clinical stage in both cohorts. These results are consistent with previous findings for pancreatic, breast and prostate carcinomas (Guo *et al.*, 2008; Liu *et al.*, 2016; Wang *et al.*, 2017), suggesting that Sox7 expression is unable to distinguish between PCa patients with different clinical stages, such as invasion and metastasis. However, another PCa study reported that Sox7 immunostaining in metastatic PCa tissues is higher than in non-metastatic tissues (Zhong *et al.*, 2012) and there was a negative association between Sox7 immunostaining and clinical stage of the liver cancer (Wang *et al.*, 2017). Multiple reasons could explain the differences between the Sox7 results, including sample collection, antibodies used, as well as cancer type.

To our knowledge, this study is the first IHC study that showed an association between nuclear and cytoplasmic Sox7 staining and the risk of relapse, using IHC. However, this result is supported by a previous prostate study by Zhong, *et al.* that reported that Sox7 mRNA and protein levels were decreased in castration-resistant regrowth tumour mouse models (CR) compared to castration-induced regression nadir (ND) and androgen-dependent growth (AD) tumours, using RT-PCR and western blot analysis (Zhong *et al.*, 2012). They proposed that downregulation of Sox7 might play a potential role in increasing risk of biochemical relapse.

Further work could be carried out to extend the analysis of Sox7 expression reported in this thesis. Another method, such as RNAscope® (described in Chapter 2), might be used to determine the Sox7 mRNA level in both normal and malignant prostate tissue samples from

both cohorts. This would provide an independent method to confirm the IHC findings. In addition, the present study suggests that there is a negative association between Sox7 immunostaining and risk of biochemical relapse. However, there are still many unanswered questions about this association, because the sample size of the Bath cohort is small and the TMA cohort does not have clinical data regarding the PCa relapse and non- relapse. It would be very interesting to assess the expression of Sox7 at protein and mRNA levels, using IHC and RNAscope in a large TMA cohort which has clinical information about a risk of PCa biochemical relapse (see Chapter 8 for more details).

5.3.3 The regulation of Sox7 expression in prostate cancer

There are different mechanisms, including promoter hypermethylation and allelic deletion, that have been suggested to suppress the expression of Sox7 in PCa (Guo *et al.*, 2008). A previous report demonstrated that a frequently methylated CPG island, which is located in the promoter region of Sox7, plays an important role in regulating the expression of Sox7 (Stovall *et al.*, 2014). As a result of a tumour specific promoter hypermethylation which is detected in 48% of PCa and 44% of PCa cell lines, Sox7 level was reduced significantly in PCa and PCa cell lines compared to NP and NP cell lines, respectively using methylation-specific PCR (MSP) (Guo *et al.*, 2008). Furthermore, another study has identified that reduction of Sox7 mRNA and protein levels in breast cancer cell lines compared to normal breast cell lines was found to be due to Sox7 promoter hypermethylation which was higher in breast cancer cell lines compared to normal breast cell lines (Stovall *et al.*, 2013).

In addition to the promoter hypermethylation, Sox7 gene deletion is another mechanism for Sox7 loss in cancers, including PCa. Previous studies have been indicated that the Sox7 gene is located in a short arm of chromosome 8 that is a frequently deleted in a number of cancers, including PCa (Oba *et al.*, 2001; Stovall *et al.*, 2014). Sox7 deletion has also been reported in multiple non – small cell lung cancer (NSCLC) cell lines (Hayano *et al.*, 2013). Another study reported that allelic loss was found in 18 PCa samples (61%) and also found that 64% of PCa (7/11) had a combination of promoter methylation and allelic loss (Guo *et al.*, 2008).

Therefore, the downregulation of Sox7 seen in this study is likely to be due to a combination of promoter hypermethylation and allelic loss.

5.3.4 The function of Sox7 in prostate cancer

Sox7 is a transcription factor that has been proposed to be a tumour suppressor in multiple cancers, including PCa and previous studies have been suggested that Sox7 expression might inhibit cell proliferation, migration, invasion and colony formation in PCa and breast cancer (Guo *et al.*, 2008).

The Sox7 protein consists of 338 amino acids and has a β-catenin interaction site on its C-terminal domain (Takash *et al.*, 2001; Guo *et al.*, 2008; Stovall *et al.*, 2014). It has been reported that Sox7 can inhibit β-catenin /T cell factor (TCF) regulated transcription (CRT) (Takash *et al.*, 2001). Another study showed that Sox7 can physically interact through its β-catenin binding motif with β-catenin protein and then inhibits its activity (Guo *et al.*, 2008; Chang *et al.*, 2013; Liu *et al.*, 2016), suggesting Sox7 negatively regulates active β-catenin. There was a significant reduction in active β-catenin in transfected cells with Sox7, but transfected cells were found to have the same expression level of β-catenin (Guo *et al.*, 2008), suggesting Sox7 depletes active, but not total β-catenin. This argues that a reduction in Sox7 might cause an increase in active β-catenin that would counteract some of the loss of total β-catenin expression seen in PCa (see Chapter 3) and so contribute to tumour formation and progression.

Interestingly, a study on breast cancer demonstrated that Sox7 and Axin-2 (Axin inhibition protein-2) expression is reduced in breast cancer, compared to the normal breast and they have a cooperative role in regulating Wnt/ β-catenin signalling pathway (Liu *et al.*, 2016). For future work, it would be very interesting to investigate Axin 2 expression in normal and malignant prostate tissues and to determine if there is an association between Axin 2 and histopathological and clinical parameters of PCa, including primary Gleason grade, clinical stage and biochemical relapse.

5.3.5 Conclusion

In conclusion, Sox7 was found to be downregulated in PCa and its expression negatively associated with primary Gleason grade and biochemical recurrence. Sox7 downregulation may be a potential indicator for aggressive PCa progression and risk of relapse. Further studies, however, are needed to confirm the role of Sox7 in risk of the biochemical relapse and elucidate its molecular function in normal and cancerous prostate tissues. Additional future work is considered in the final discussion.

CHAPTER SIX

IDENTIFICATION AND ASSESSMENT OF SALL4 EXPRESSION IN PROSTATE TISSUES FROM THE BATH AND TMA COHORTS

6. Identification and Assessment of Sall4 expression in prostate tissues from the Bath and TMA cohorts

6.1 Introduction

One of the candidate biomarkers that has been identified for analysis was Sall4. This chapter focuses on Sall4 and describes why the protein was selected for analysis, describes the possible role of RNAscope® in cancer and presents all the experimental data that was collected for the protein.

6.1.1 Identification of Sall4 using literature searching

Sall4 is a zinc transcription factor found to be expressed in ESCs and during the development of embryos, but its expression level after birth is observed to be decreased in most of normal human adult tissues. Sall4, however, is found to be reactivated in a number of cancers (Zhang *et al*., 2015; Tatetsu *et al*., 2016). Of particular importance for this study, altered Sall4 levels have been suggested to play a role in PCa progression and relapse (described below and summarised in Table 6.1).

A previous IHC study showed nuclear Sall4 staining in a subset of non-small lung carcinoma (33.8%) and only 10% of normal lung tissues (1/10 cases) (Rodriguez *et al.*, 2014). In colorectal carcinoma, Sall4 mRNA was found to be expressed two times higher than in normal colon tissues, using real-time PCR (Forghanifard *et al.*, 2013). In contrast, another study showed a reduction of nuclear and cytoplasmic Sall4 staining in colorectal carcinoma compared to normal colon tissues (Hao *et al.*, 2016). In PCa, according to our knowledge, the association between Sall4 staining in normal and malignant prostate tissue has not been extensively investigated, however a couple of studies on other cancers have included small numbers of prostate tissue samples. For example, a previous study on non- small cell lung carcinomas used a TMA with 112 normal tissues as controls, including 7 NP tissues and showed a weak nuclear Sall4 staining in 57% of NP tissues (4/7) (Rodriguez *et al.*, 2014). Another study on metastatic germ & non-germ cell tumours showed negative Sall4 staining in all metastatic PCa samples (12 cases only) (Cao *et al.*, 2009). Therefore, most of the studies on other tumours, and the limited evidence relating to PCa suggest that increased nuclear Sall4 staining might be associated with PCa.

In terms of a link with PCa histopathological features, including primary Gleason grade, clinical stage and biochemical relapse, according to our knowledge, there is no specific study

that investigates a link between Sall4 expression and PCa histopathological parameters. However, Sall4 association with the histopathological parameters has been studied in other types of cancer tissues, including lung and colorectal carcinomas (Rodriguez *et al.*, 2014; Hao *et al.*, 2016). An IHC study on non-small cell lung carcinoma reported that nuclear Sall4 staining was significantly stronger in high grade primary lung adenocarcinomas compared to low grade (Rodriguez *et al.*, 2014). In contrast, another study showed that there was a negative association between Sall4 expression and colorectal carcinoma grades (Hao *et al.*, 2016). Finally, other studies have reported that Sall4 expression is not significantly associated with grades of lung cancer (Gautam *et al.*, 2015; Han *et al.*, 2014), particularly primary squamous cell carcinoma (Rodriguez *et al.*, 2014) and hepatocellular carcinoma (Han *et al.*, 2014). Therefore, there is contradictory evidence from other tumours relating to the relationship between Sall4 and cancer grades.

The published evidence is also complicated and often at least partially contradictory for the association between Sall4 expression and clinical stage. Nuclear Sall4 staining was negatively associated with primary lung squamous cell carcinoma stage T (pT1 vs pT2) (Rodriguez *et al.*, 2014). In contrast, in colorectal carcinoma, Sall4 mRNA expression was significantly increased in a tumour metastatic to lymph nodes compared to non-metastatic tissues (Forghanifard *et al.*, 2013; Rodriguez *et al.*, 2014). Other studies, however, showed no significant association between nuclear Sall4 expression and stage of lung (Rodriguez *et al.*, 2014; Gautam *et al.*, 2015) and hepatocellular carcinomas (Han *et al.*, 2014). Therefore, there is no significant evidence to suggest that an alteration in nuclear Sall4 expression might be linked to PCa stages.

Finally, in terms of a link with relapse, there is only one study which found that Sall4's level in serum increased significantly in HCC recurrence patients compared to those with non-recurrence (Han *et al.*, 2014). Therefore, there is very little evidence to suggest that Sall4 expression might be linked to PCa relapses.

Given the evidence described above, it was decided to examine nuclear Sall4 staining in prostate tissues, including recurrence and non-recurrence. The hypothesis used predicted that nuclear Sall4 staining will be increased in PCa, but will not be associated with primary Gleason grades, stage and relapse. The key publications and hypothesis for Sall4 are summarised in Table 6.1.

Table 6. 1 Shows the key findings and hypothesis of Sall4.

Name & type of Protein	Key publications	Hypothesis
Sall4 **A zinc finger transcription factor**	❖ Sall4 was expressed strongly in the nucleus of metastatic germ cell tumours, whereas, a small number of metastatic PCa (only 12 cases) showed a negative Sall4 expression (Cao *et al.*, 2009). ❖ Sall4 mRNA level in colorectal carcinoma was two times higher compared to normal colon tissues and was significantly associated with lymph node metastasis, using a real-time PCR (Forghanifard *et al.*, 2013). ❖ A subset of non-small lung carcinoma showed nuclear expression of Sall4, and this expression of Sall4 was significantly stronger in high grades of primary lung adenocarcinoma, whereas, there was no a significant associated between Sall4 expression and primary squamous cell carcinoma grades. Sall4 was also expressed weakly in four from seven NP tissues (Rodriguez *et al.*, 2014). ❖ Sall4 serum level was increased significantly in hepatocellular carcinoma compared to normal controls and was also significantly increased in HCC recurrence compared to non- recurrence (Han *et al.*, 2014; Hang *et al.*, 2012). ❖ Sall4 cytoplasmic and membranous expression was high in lung cancer compared to normal, and there was not an association between Sall4 expression and histopathological parameters, including grades and stages (Gautam *et al.*, 2015). ❖ Nuclear and cytoplasmic Sall4 expression was significantly reduced in colorectal carcinoma compared to normal colon and was negatively associated with increasing grade, whereas, it was positively associated with advanced stages (Hao *et al.*, 2016).	❖ Nuclear Sall4 staining will be increased in PCa compared to NP tissues. ❖ Nuclear Sall4 staining will be not associated with grade, stage and relapse

6.1.2 The role of RNAscope ® in prostate cancer

In situ hybridization (ISH) is a technique used to detect DNA and RNA levels in specific cell populations and tissue samples (Jensen, 2014; Grabinski et al., 2015). In clinical use, ISH is commonly used to detect DNA biomarkers, whereas, RNA detection is rare (Wang *et al*., 2012) as standard ISH has some difficulties in detecting RNA in samples (Jensen, 2014). These difficulties include non-specific binding and low sensitivity (Ambinder and Mann, 1994), especially in detecting an RNA with a low level of expression (Grabinski *et al.*, 2015).

To overcome the limitations with traditional ISH a previous study developed a novel RNA ISH technique called RNAscope® (Wang *et al.*, 2012) that represents a simple nonradioisotopic ISH method to detect mRNA in cells or tissues, including FFPE sections (Wang *et al.*, 2012; Grabinski *et al.*, 2015). It can be used to detect either single or multiple

RNA species using chromogenic or fluorescent methods (Wang *et al.*, 2012; Grabinski *et al.*, 2015). It uses twenty double Z probes which bind directly to the target mRNA and then this interaction is amplified many times using a cascade of a preamplifier, amplifier, and label (Wang *et al.*, 2012) (Figure 1.6). This design helps to improve the specificity and sensitivity of the results because of its amplification of the target-specific signals without amplifying background staining (Wang *et al.*, 2012; Grabinski *et al.*, 2015). RNAscope® represents a significant advance in RNA methodology (Wang *et al.*, 2012). In addition, a previous study on thick free-floating rodent brain sections and primary neuronal cultures suggested that a combination of RNAscope® and IHC is a useful method that can be used to detect RNA and protein in the same samples (Grabinski *et al.*, 2015).

Clinically, RNAscope® has been used to detect Epstein-Barr virus (EBV) -related diseases (Ambinder and Mann, 1994; Gulley, 2001). However, it's still used rarely (Bingham *et al.*, 2016; Wang *et al.*, 2012), particularly in cancer fields, but it has the potential to have an important role in the future diagnosis of PCa and is used in this chapter.

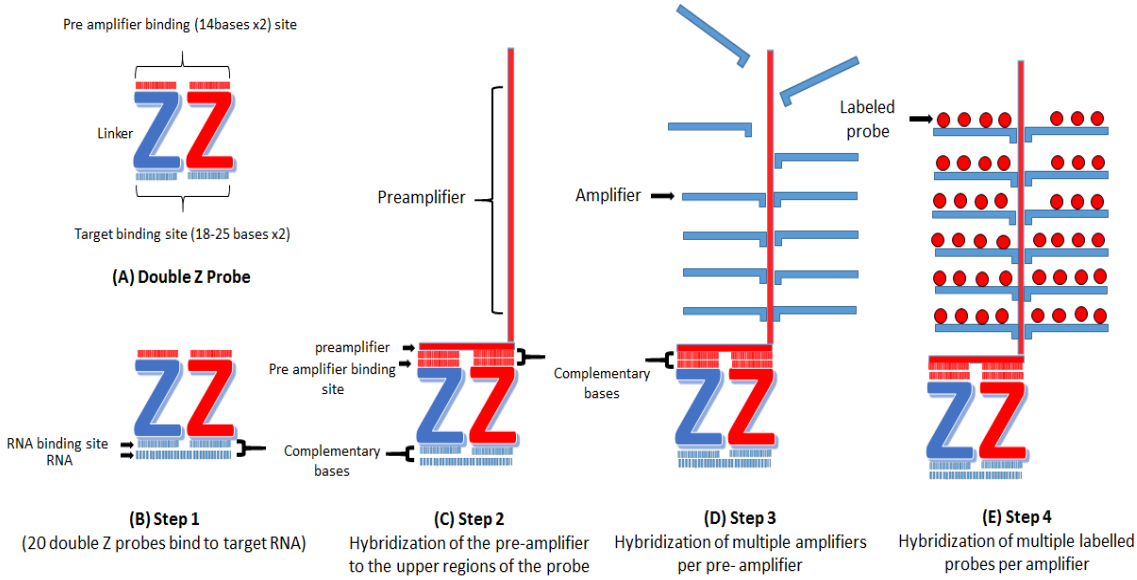

Figure 6. 1 Principle of RNAscope®. (A) RNAscope probe design which consists of 20 double Z probes targeting a region of 1000 based and each Z target has three elements: The lower region which is complementary to the target RNA for the target, two upper regions which consist of 28 base binding sites and link to preamplifier and spacer sequence which links the lower and upper regions. (B) Step 1: The double Z paris bind to RNA target in the lower region of the probe (C) Step 2: The preamplifier hybridizes to the upper regions of the Z probe (D) Step 3: Hybridization of multiple amplifiers per preamplifier. (E) Step 4: Hybridization of multiple labelled probed per amplifier. These serial steps of hybridization, including 20 double Z probe, multiple amplifiers and multiple labelled probes leads to form thousands of labelled probes per RNA target.

6.1.3 Aims

The goal of this chapter is to test the hypothesis, described above by carrying out the following:

1. To detect the expression levels of Sall4 in normal and malignant prostate tissues from the Bath and TMA cohorts using two independent antibodies.
2. To establish if the expression of Sall4 correlates with clinical features of PCa, including primary Gleason grade, clinical stage and biochemical recurrence.
3. To confirm the specificity of Sall4 staining pattern and IHC results by repeating the experiments with a second independent antibody.
4. To confirm the specificity of the Sall4 IHC staining by using RNAscope® to measure the expression of Sall4 mRNA and comparing the mRNA expression pattern to those produced using IHC.

6.2 Results

6.2.1 Testing of antigen retrieval methods

Having chosen Sall4 as a candidate biomarker, the next step was to assess its expression using IHC. Before this could be carried out, different types of antigen retrieval, including citrate and Tris/EDTA were tested to establish which gave the best staining for Sall4.

The IHC was carried out with an anti-Sall4 antibody from Novus that is referred to here as the anti-Sall4 A antibody. IHC staining with Tris/EDTA buffer showed nuclear anti-Sall4 A staining in prostate tissues from the Bath cohort (Figure 6.2 B), whereas, the tissue section from the same region from prostate tissue showed negative anti-Sall4 A staining when using citrate buffer (Figure 6.2 A). This study chose Tris/EDTA buffer as antigen retrieval for anti-Sall4 A because it showed a clear nuclear anti-Sall4 A staining.

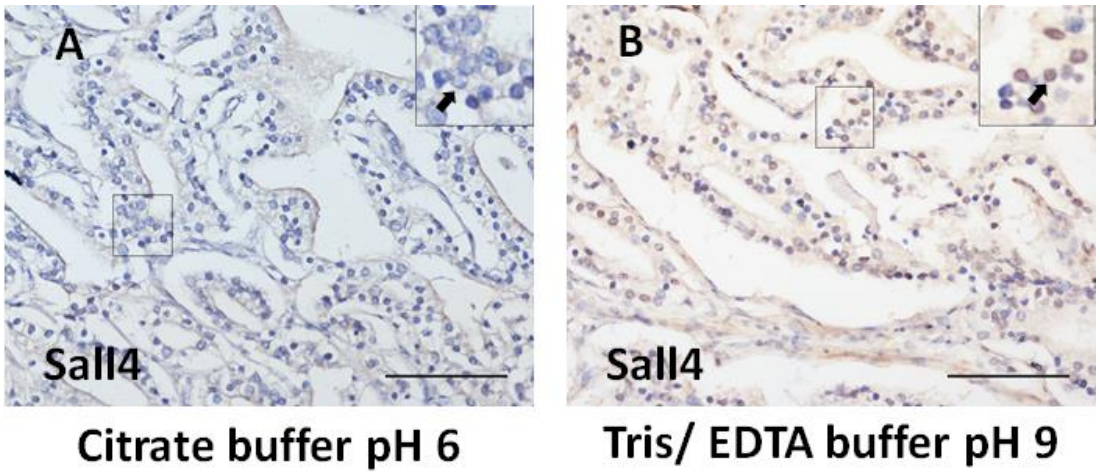

Figure 6. 2 Expression of anti-Sall4 A after different antigen retrieval methods. (A) A negative anti- Sall4 A staining (Black arrow) was observed in prostate tissues from the Bath cohort, using citrate buffer. (B) Strong nuclear anti-Sall4 A staining (Black arrow) was observed in prostate tissue, using Tris/EDTA buffer. IHC staining with Tris/EDTA buffer showed a clear nuclear anti-Sall4 A staining, whereas citrate buffer showed negative staining in a section of the similar region of prostate tissues. Scale bars=100μm with inserts at 2x zoom.

6.2.2 Expression of Sall4 in the Bath cohort, using the anti-Sall4 A antibody

To test the hypothesis that nuclear Sall4 staining will be increased in PCa but will be not associated with PCa clinical features such as Gleason grade, clinical stage and biochemical relapse, IHC was carried out with the Bath cohort of prostate tissue samples using the anti-Sall4 A antibody.

6.2.2.1 Immunohistochemical staining of anti-Sall4 A in the Bath prostate samples

IHC result showed nuclear anti-Sall4 A staining in NP tissues, which ranged from strong (Figure 6.3 A, arrow) to moderate (Figure 6.3 B, arrow). Nuclear anti-Sall4 A staining was also observed in PCa tissues and the intensity of signal varied widely, from strong and widespread (Figure 6.3 C, arrow), moderate (Figure 6.3 D&E, arrows) to weak and scattered (Figure 6.3 F, arrow). Cytoplasmic anti-Sall4 A staining was also detected in some normal and malignant prostate tissues, with variable levels between cases, ranging from moderate (Figure 6.3 E, arrowhead) to weak (Figure 6.3 A, arrowhead) or negative (Figure 6.3, C). Sall4 is expressed in the nucleus of the spermatogonia cells of testis tissues (Eildermann *et al.*, 2012) and so this study used normal testis tissues as a positive control for anti-Sall4 A and as expected IHC showed nuclear anti-Sall4 A staining in spermatogonia cells (Figure 6.3 G, arrow). A no primary antibody negative control showed no significant background staining in prostate tissue (Figure 6.3 H, arrow).

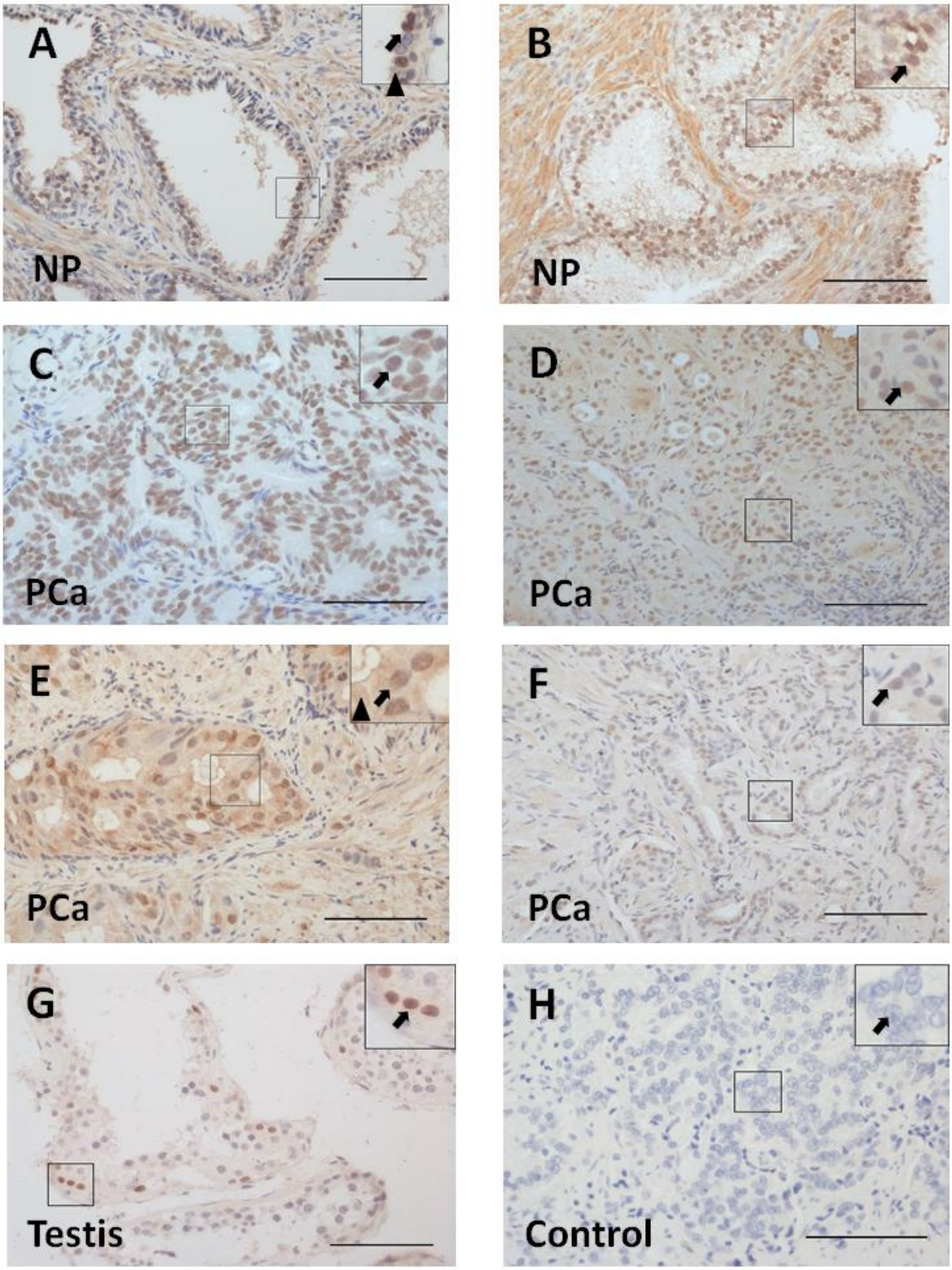

Figure 6. 3 Sall4 A staining in samples from the Bath cohort, using anti-Sall4 A antibody. Anti-Sall4 A staining was found heterogeneously in both normal and malignant tissues of the prostate. (A) Strong nuclear (Black arrow) and weak cytoplasmic (Black arrowhead) anti-Sall4 A staining in NP. (B) Moderate nuclear (Black arrow) anti-Sall4 A staining in NP. (C) Strong nuclear anti-Sall4 A staining (Black arrow) in PCa. (D) Moderate nuclear anti-Sall4 A (Black arrow) staining in PCa. (E) Moderate nuclear (Black arrow) and cytoplasmic (Black arrowhead) anti-Sall4 A staining in PCa. (F) Weak nuclear anti-Sall4 A staining (Black arrow) in PCa. (H) Positive control. Strong nuclear anti-Sall4 A staining (Black arrow) in spermatogonia cells of normal testis tissues. (H) Negative control (no primary antibody added) was free from background staining (Black arrow) in PCa. Scale bars=100µm with inserts at 2x zoom.

6.2.2.2 Association between anti-Sall4 A immunostaining and histopathological parameters of prostate cancer in the Bath cohort

In order to evaluate if there was an association between anti-Sall4 A staining in normal vs malignant prostate tissues, primary Gleason grade, clinical stage and biochemical relapse, nuclear anti-Sall4 A staining was quantified using the H-score system (described in Chapter 2.3.1) and then compared to histopathological and clinical features of PCa using the clinical data available for the Bath cohort. The range of nuclear anti-Sall4 A H-score varied between 0 - 160 (Figure 6.4). This study excluded cytoplasmic anti-Sall4 staining for further analysis because Sall4 is a transcription factor and is expected to function in the nucleus of the prostate tissue and the majority of the previous studies have focused on nuclear Sall4 staining.

The first analysis looked at the staining of anti-Sall4 A in normal vs. malignant prostate tissues. Quantification of the IHC staining showed that nuclear anti-Sall4 A staining was reduced significantly in PCa compared to NP tissues (P= 0.0266) (Figure 6.4 A & Table 6.2). There was also a significant reduction in the mean of nuclear anti-Sall4 A scores when comparing PCa tissues with a primary Gleason grade 4 to those with a grade 3 (p= 0.0455) (Figure 6.4 B & Table 6.2). In contrast, the current study showed no significant association between nuclear anti-Sall4 A staining and clinical stage T (p= 0.9072) or biochemical recurrence (p= 0.7311) (Table 6.2)

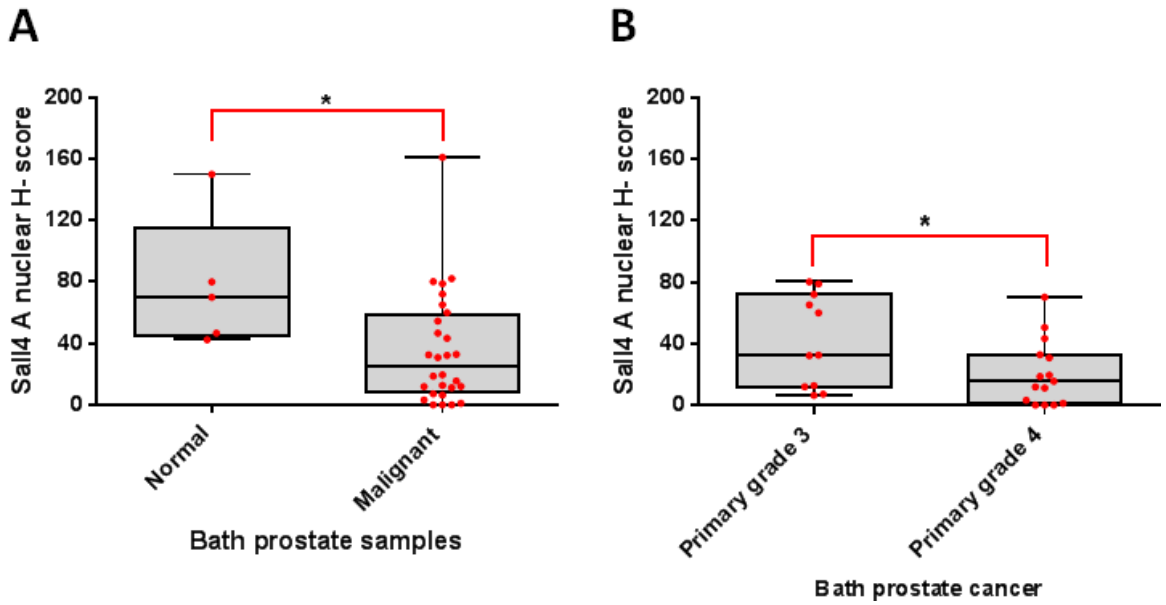

Figure 6. 4 Quantification of nuclear anti-Sall4 A staining in both normal and malignant Bath prostate tissues. Immunohistochemical staining of nuclear anti-Sall4 A was quantified in the Bath cohort using H-score system. (A) Nuclear anti-Sall4 A staining was significantly reduced in PCa compared to NP tissues (p=0.0266). (B) Nuclear anti-Sall4 staining was reduced significantly in primary Gleason grade 4 tissues compared to grade 3 tissues (p= 0.0455). Data represent the mean of five random images pre-case. Unpaired t-tests were conducted

to determine the statistical difference for each condition. NP (n=5), PCa (n=28), grade 3 (n=11) & grade 4 (n=15).

Table 6. 2 Nuclear anti-Sall4 A staining results with the Bath cohort clinical data.

Comparison	Nuclear anti-Sall4 A staining	
	Results	**p. value**
Normal vs malignant	Lower in malignant	0.0266
Primary Gleason grades (3 & 4)	Lower in high grade	0.0455
Clinical Stage (T1-2&T3-4)	No statistically significant difference	0.9072
5 years Biochemical recurrence	No statistically significant difference	0. 7311

In summary, nuclear anti-Sall4 A staining was reduced significantly in PCa and was negatively associated with increasing primary Gleason grade, but was not associated with clinical stage T and biochemical recurrence. The Bath results, however, should be treated as preliminary data because of the small sample size.

6.2.3 Expression of Sall4 in the TMA cohort, using anti-Sall4 A antibody

Sall4 expression was then investigated on prostate tissue samples from the TMA cohort, using IHC.

6.2.3.1 Immunohistochemical staining of anti-Sall4 A in the TMA prostate samples

IHC staining of samples from the TMA showed nuclear anti-Sall4 A staining in NP tissues with variable levels of staining, ranging from strong (Figure 6.5 A, arrow) to moderate (Figure 6.5 B, arrow). Nuclear Sall4 staining was also detected in PCa tissues and the intensity of signal varied widely, from strong and widespread (Figure 6.5 C&D, arrows), moderate (Figure 6.5 E, arrow), weak (Figure 6.5 F, arrow) and negative (Figure 6.5 G, arrow). In addition, cytoplasmic anti-Sall4 A staining was also observed in normal and malignant prostate tissues, but the level varied between cases, ranging from moderate (Figure 6.5 C, arrowhead) to weak (Figure 6.5 B, D, arrowheads) or negative (Figure 6.5 G, arrow). A negative control showed no significant background staining in prostate tissue (Figure 6.5 H, arrow).

6.2.3.2 Association between anti-Sall4 A immunostaining and histopathological parameters of prostate cancer in the TMA cohort

Nuclear anti-Sall4 A staining was quantified using the H-score system and compared to normal vs. malignant prostate tissues as well as the other histopathological features of PCa, including primary Gleason grade, clinical stage and biochemical recurrence using the clinical data available for the TMA cohort. The range of nuclear anti-Sall4 A H-score in the TMA cohort varied between 2.95 to 247 (Figure 6.6).

The statistical analysis looked first at the expression of anti-Sall4 A in normal vs tumour of prostate tissues. Quantification of the IHC staining showed a significant reduction of nuclear anti-Sall4 A staining in PCa compared to NP tissues ($p= 0.0003$) (Figure 6.6 A & Table 6.3). There was also a significant difference in the mean of nuclear anti-Sall4 A H-score among primary Gleason grades ($p= <0.0001$) (Figure 6.6 B & Table 6.3), using an ANOVA test. Analysis of the IHC data, using multi comparison Tukey's tests showed nuclear anti-Sall4 A staining negatively associated with increasing primary Gleason grades. This significant reduction of nuclear anti-Sall4 A staining was shown when comparing PCa patients with a primary Gleason grade 5 to those with a grade 3 ($p=<0.0001$) or a grade 4 ($p=0.0050$) (Figure 6.6 B & Table 6.3), whereas there was no a significant difference in the mean of nuclear anti-Sall4 A score between primary Gleason grade 3 to grade 4 tissues ($p= 0.0782$) (Figure 6.6 B & Table 6.3). In contrast, nuclear anti-Sall4 A staining was not associated significantly with clinical stage T (T1-2vs T3-4) (Table 6.3).

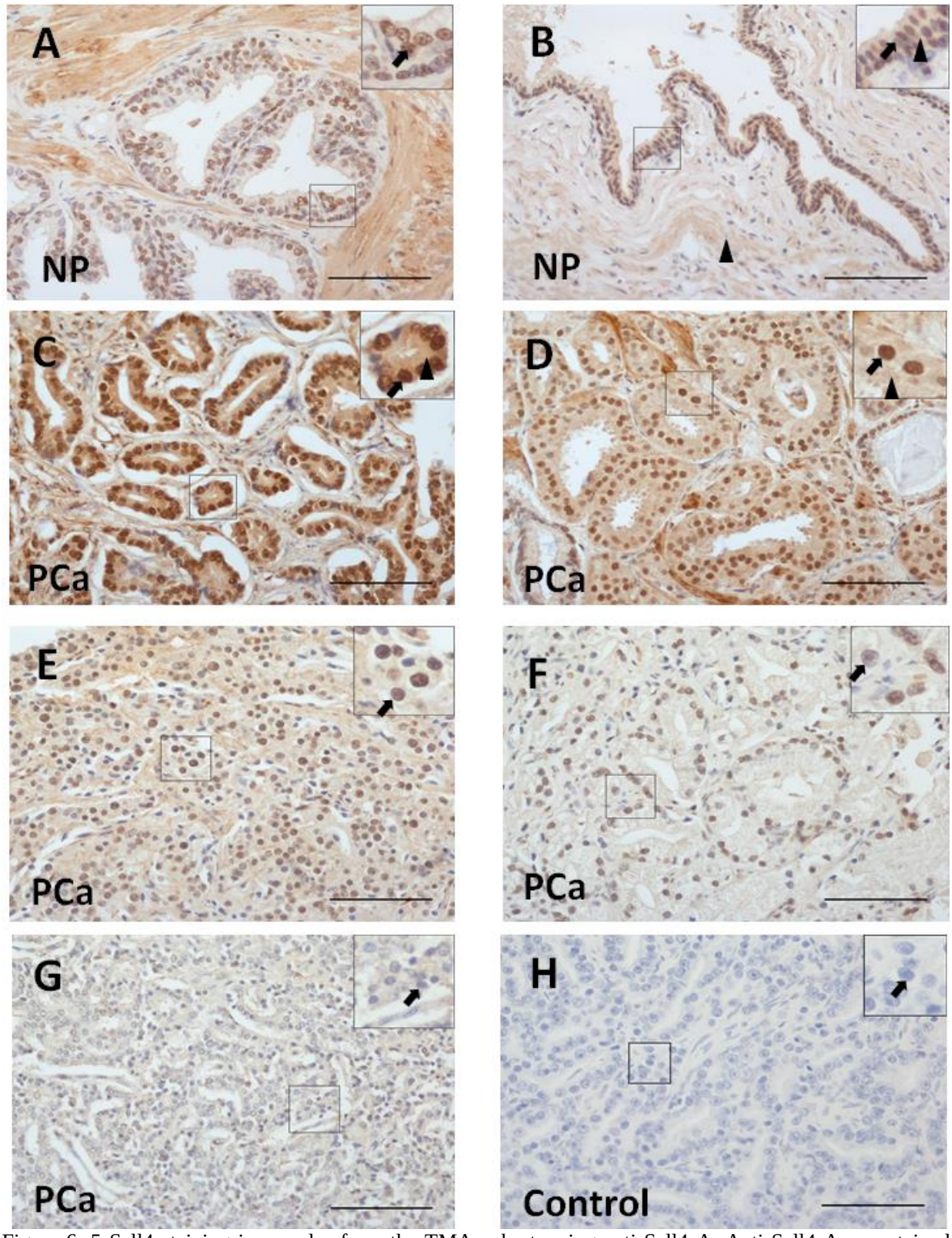

Figure 6. 5 Sall4 staining in samples from the TMA cohort, using anti-Sall4 A. Anti-Sall4 A was stained heterogeneously in both normal and malignant tissues of the prostate. (A) Strong nuclear anti- Sall4 A staining (Black arrow) in NP. (B) Strong to moderate nuclear (Black arrow) and weak cytoplasmic (Black arrowhead) anti-Sall4 A staining in NP. (C) Strong nuclear (Black arrow) and moderate cytoplasmic (Black arrowhead) anti-Sall4 A staining in PCa. (D) Strong nuclear (Black arrow) and weak cytoplasmic (Black arrowhead) anti-Sall4 A staining in PCa. (E) Moderate nuclear (Black arrow) anti-Sall4 A staining in PCa. (F) Weak nuclear anti-Sall4 A staining (Black arrow) in PCa. (G) PCa had no anti-Sall4 A staining. (H) Negative control (no primary antibody added) no detectable background staining (Black arrow) in PCa. Scale bars=100µm with inserts at 2x zoom.

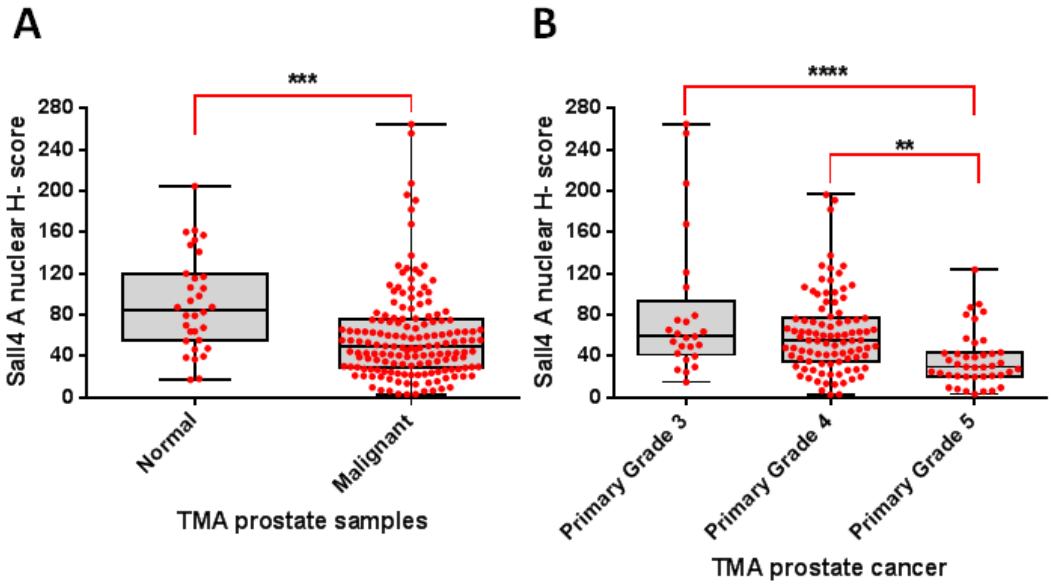

Figure 6. 6 Quantification of nuclear anti-Sall4 A staining in both normal and malignant TMA prostate tissues. Immunohistochemical staining of anti-Sall4 A was quantified in the TMA cohort using H-score system for nuclear IHC staining. (A) Nuclear anti-Sall4 A staining was significantly reduced in PCa compared to NP tissues (p=0.0003). (B) Nuclear anti-Sall4 A staining showed a significant difference among primary Gleason grades, using An ANOVA test (p=< 0.0001). Multi comparison (Tukey) tests showed a significant reduction in the mean of nuclear anti-Sall4 A staining when comparing patients with a primary Gleason grade 5 to those with a grade 3 (p=<0.0001) or grade 4 (p=0.005). There was no significant association in nuclear anti- Sall4 A staining between primary Gleason 4 and to grade 3 (p=0.0705). Data represent the mean of five random images per case. Unpaired or one-way ANOVA tests were conducted to determine the statistical difference for each set of conditions. NP (n=16), PCa (80), grade 3 (n=13), grade 4 (n=46) and grade 5 (n=18).

Table 6. 3 Nuclear anti-Sall4 A staining results with the TMA clinical data.

Comparison	**Nuclear anti-Sall4 A staining**		
	Results		**p. value**
Normal vs malignant	Lower in malignant		0.0003
Primary Gleason grades (3, 4 &5)	Lower in high Gleason grade	Anova test	<0.0001
		Grade 4 vs. Grade 3	0.0782
		Grade 5 vs. Grade 3	<0.0001
		Grade 5 vs. Grade 4	0.0050
Stage (T)	No statistically significant difference		0.1744
Stage (M)	No statistically significant difference		0.2207
Stage (N)	No statistically significant difference		0.6314

In summary, nuclear anti-Sall4 A staining of the TMA samples was reduced significantly in PCa compared to NP and was negatively associated with increasing primary Gleason grade, but not with the stage (TNM). This result was consistent with Sall4 A results from the Bath cohort.

6.2.4 Sall4 Antibody validation

In order to show that the anti-Sall4 A staining described above was accurately reporting changes in Sall4 protein expression, the results should be repeated with an independent antibody. To achieve this two possible additional anti-Sall4 antibodies were identified, a rabbit monoclonal and a rabbit polyclonal from Abcam that are referred to here as anti-Sall4 B and anti-Sall4 C, respectively.

The ideal situation would have been to have the second antibody raised to a distinct part of the protein, however, this was not possible as any new antibodies identified were all raised using immunogens from a similar region of Sall4 (Figure 6.7). The original antibody, human anti-Sall4 A, is a mouse monoclonal antibody raised with an immunogen that was a partial recombinant protein fragment of the C-terminal of Sall4 protein (Figure 6.6 A, 1). Human anti-Sall4 B is a rabbit monoclonal with a recombinant immunogen from within the same region of Sall4 protein (Figure 6.7 A, 2). Anti-Sall4 C is a rabbit polyclonal antibody raised against residues 1000 to the C- terminus of mouse Sall4 protein (Figure 6.7 B). Mouse anti-Sall4 C has a few different amino acids to the human anti-Sall4 A&B (Figure 6.7 B,1 black Bold), but the antibody is reported to react with human tissues (https://www.abcam.com/Sall4-antibody-chip-grade-ab29112.html). Therefore, although not raised to different regions of the protein, the three antibodies were raised independently (shown by the fact they have different hosts or clonality) and so were chosen for testing on normal testis and prostate tissues from the Bath cohort.

A. Sall4 human protein sequence:

```
   1 msrrkqakpq hinseedqge qqpqqqtpef adaapaapaa gelgapvnhp gndevasede
  61 atvkrlrree thvcekccae ffsisefleh kknctknppv limndsegpv psedfsgavl
 121 shqptspgsk dchrenggss edmkekpdae svvylkteta lpptpqdisy lakgkvantn
 181 vtlqalrgtk vavnqrsada lpapvpgans ipwvleqilc lqqqqlqqiq lteqiriqvn
 241 mwashalhss gagadtlktl gshmsqqvsa avallsqkag sqglsldalk qaklphanip
 301 satsslspgl apftlkpdgt rvlpnvmsrl psallpqapg svlfqspfst valdtskkgk
 361 gkppnisavd vkpkdeaaly khkckycskv fgtdsslqih lrshtgerpf vcsvcghrft
 421 tkgnlkvhfh rhpqvkanpq lfaefqdkva agngipyals vpdpidepsl sldskpvlvt
 481 tsvglpqnls sgtnpkdltg gslpgdlqpg pspseseggpt lpgvgpnyns praggfqgsg
 541 tpepgsetlk lqqlvenidk attdpnecli chrvlscqss lkmhyrthtg erpfqckicg
 601 rafstkgnlk thlgvhrtnt siktqhscpi cqkkftnavm lqqhirmhmg gqipntplpe
 661 npcdftgsep mtvgengstg aichddvies idveevssqe apsssskvpt plpsihsasp
 721 tlgfammasl dapgkvgpap fnlqrqgsre ngsvesdglt ndssslmgdq eyqsrspdil
 781 ettsfqalsp ansqaesiks kspdagskae ssensrteme grsslpstfi rapptyvkve
 841 vpgtfvgpst lspgmtplla aqprrqakqh gctrcgknfs sasalqiher thtgekpfvc
 901 nicgrafttk gnlkvhymth gannnsarrg rklaientma llgtdgkrvs eifpkeilap
 961 svnvdpvvwn qytsmlnggl avktneisvi qsggvptlpv slgatsvvnn atvskmdgsq
1021 sgisadvekp satdgvpkhq fphfleenki avs
```

1. **Sall4 Mouse monoclonal (Sall4 A)**

```
 901 nicgrafttk gnlkvhymth gannnsarrg rklaientma llgtdgkrvs eifpkeilap
 961 svnvdpvvwn qytsmlnggl avktneisvi qsggvptlpv slgatsvvnn atvskmdgsq
```

```
1021 sgisadvekp satdgvpkhq fphfleenki avs
```

2. **Sall4 Rabbit monoclonal (Sall4 B)**

```
 901 nicgrafttk gnlkvhymth gannnsarrg rklaientma llgtdgkrvs eifpkeilap
 961 svnvdpvvwn qytsmlnggl avktneisvi qsggvptlpv slgatsvvnn atvskmdgsq
1021 sgisadvekp satdgvpkhq fphfleenki avs
```

B. Sall4 Mouse protein sequence:

```
   1 msrrkqakpq hinseedqge qpqqlpspdl aealaaeepg apvnspgncd easedsipvk
  61 rprredthic nkccaeffsl sefmehkksc tktppvlimn dsegpvpsed fsraalshql
 121 gspsnkdslq engsssgdlk klgtdsilyl kteatqpstp qdisylpkgk vantnvtlqa
 181 lrgtkvavnq rgaeapmapm paaqgipwvl eqilclqqqq lqqiqlteqi rvqvnmwaah
 241 alhsgvagad tlkalsshvs qqvsvsqqvs aavallsqka snpalsldal kqaklphasv
 301 psaasplssg ltsftlkpdg trvlpnfvsr lpsallpqtp gsvllqspfs avtldqskkg
 361 kgkpqnlsas asvldvkakd evvlgkhkcr ycpkvfgtds slqihlrsht gerpyvcpic
 421 ghrfttkgnl kvhlqrhpev kanpqllaef qdkgavsaas hyalpvpvpa desslsvdae
 481 pvpvtgtpsl glpqkltsgp nsrdlmggsl pndmqpgpsp eseaglpllg vgmihnppka
 541 ggfqgtgape sgsetlklqq lvenidkatt dpneclichr vlscqsslkm hyrthtgerp
 601 fqckicgraf stkgnlkthl gvhrtnttvk tqhscpicqk kftnavmlqq hirmhmggqi
 661 pntplpespc dftapepvav sengsasgvc qddaaegmea eevcsqdvps gpstvslpvp
 721 sahlaspslg fsvlasldtq gkgalpalal qrqssrenss leggdtgpan dssllvgdqe
 781 cqsrspdate tmcyqavspa nsqagsvksr speghkaegv escrvdtegr tslpptfira
 841 qptfvkvevp gtfvgppsmp sgmpplasq pqprrqakqh cctrcgknfs sasalqiher
 901 thtgekpfvc nicgrafttk gnlkvhymth gannnsarrg rklaienpma alsaegkrap
 961 evfskellsp avsvdpaswn qytsvlnggl amktneisvi qsggiptlpv slgassvvsn
1021 gtiskldgsq tgvsmpmsgn geklavpdgm akhqfphfle enkiavs
```

1. **Sall4 Rabbit polyclonal (Sall4 C)s**

```
 961 evfskellsp avsvdpaswn qytsvlnggl amktneisvi qsggiptlpv slgassvvsn
1021 gtiskldgsq tgvsmpmsgn geklavpdgm akhqfphfle enkiavs
```

Figure 6. 7 Human and mouse Sall4 proteins sequence with the immunogenic parts of three different antibodies. (A) Human Sall4 protein consists of 1053 amino acid and the antibodies raised independently as they have a different host or clonality. (1) Sall4 mouse monoclonal (anti-Sall4 A) immunogen is a partial recombinant protein with SGT tag (Grey highlighted). (2) Sall4 rabbit monoclonal (anti-Sall4 B) immunogen is a recombinant fragment within Human Sall4 amino acid 950 to C- terminus (Grey highlighted). (B) Anti-Sall4 C is a rabbit polyclonal antibody raised against residues 1000 to the C- terminus of mouse Sall4 protein. Anti-Sall4 C immunogen is a synthetic peptide conjugated to KLH derived from residues 1000 to C- terminus of mouse Sall4 (Grey highlighted) and the different amino acids from Human Sall4 are shown bold.

6.2.4.1 Immunohistochemical staining patterns of Sall4 antibodies on testis and prostate tissues from the Bath cohort

IHC was carried out using the three different anti-Sall4 antibodies (anti-Sall4 A, B&C) on the section from similar regions of testis and prostate tissues from the Bath cohort. In testis tissue, the three antibodies (anti-Sall4 A, B&C) showed strong nuclear Sall4 staining in the spermatogonia cells (Figure 6.8 A, B&C, arrows). In addition to the nuclear staining, anti-Sall4 C was also observed in the cytoplasm of spermatogonia cells (Figure 6.8 C, arrowheads). In prostate tissues, anti-Sall4 A& B staining was observed in nuclei of glandular epithelial cells (Figure 6.8 D, E, G &H, arrows). In contrast, a predominant cytoplasmic anti-Sall4 C staining (Figure 6.8 F&I, arrowheads), with some weak nuclear staining, was observed in prostate tissue. It can be seen that the human anti-Sall4 A& B

antibodies had very similar staining patterns in both testis and prostate tissue samples, whereas, anti-Sall4 C staining was less similar. For this reason, anti-Sall4 B, and not anti-Sall4 C, was used for further analysis.

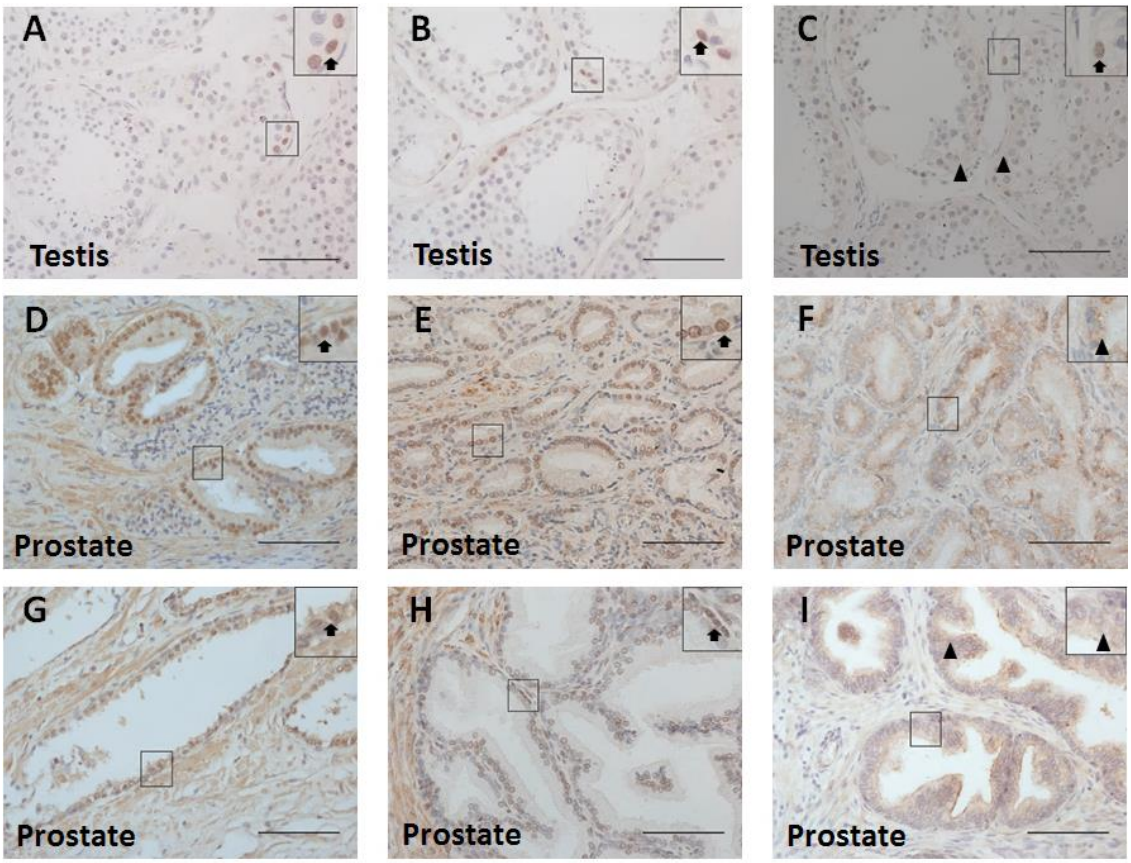

Figure 6. 8 Immunohistochemical staining using three distinct Sall4 antibodies in testis and prostate tissue samples. (A) Strong nuclear anti-Sall4 A staining (Black arrow) in spermatogonia cells of the normal testis tissues. (B) Strong nuclear anti-Sall4 B staining (Black arrow) in spermatogonia cells of normal testis tissues. (C) Moderate nuclear (Black arrow) and weak cytoplasmic (Black arrowheads) anti-Sall4 C staining in spermatogonia cells of normal testis tissues. (D) Strong nuclear anti-Sall4 A staining (Black arrow) in the glandular regions of prostate tissue. (E) Strong nuclear anti-Sall4 B staining (Black arrow) in the glandular regions of prostate tissue. (F) Moderate cytoplasmic anti-Sall4 C staining (Black arrowhead) in the glandular regions of prostate tissue. (G) Moderate nuclear anti-Sall4 A staining (Black arrow) in the glandular regions of prostate tissue. (H) Moderate cytoplasmic anti-Sall4 C staining (Black arrowhead) in the glandular regions of prostate tissue. (I) Moderate cytoplasmic anti-Sall4 C staining (Black arrowhead) in the glandular regions of prostate tissue. Both human anti-Sall4 A&B antibodies showed the same staining patterns in testis and prostate tissues. Mouse anti-Sall4 C showed predominate cytoplasmic staining pattern in testis and prostate tissues. Scale bars—100 μm with inserts at 2x zoom.

6.2.5 Expression of Sall4 in the Bath cohort, using anti-Sall4 B Antibody

After confirming that human anti-Sall4 A & B antibodies had similar staining patterns in testis and prostate tissues from the Bath cohort, and to increase the confidence in the anti-Sall4 A IHC result; anti-Sall4 B staining was investigated on prostate tissue samples from the Bath cohort, using IHC.

6.2.5.1 Anti-Sall4 B staining in the normal and malignant Bath prostate samples

The IHC staining showed no significant staining for Sall4 in the nucleus of NP tissues, using anti-Sall4 B (Figure 6.9 A, arrow). In contrast, nuclear anti-Sall4 B staining was observed in PCa tissues and the intensity of signal varied widely, from strong and widespread (Figure 6.9 B&C, arrows), moderate (Figure 6.9 D, arrow), weak and scattered (Figure 6.9 E&F, arrows) and negative. In addition to the nuclear staining, weak cytoplasmic anti-Sall4 B staining was also found in a few cases of PCa (Figure 6.9 B & E, arrowheads). Strong nuclear anti-Sall4 B staining was detected in the positive control spermatogonia cells of normal testis tissue (Figure 6.9 G, arrow). A negative control showed no detectable background staining in prostate tissue (Figure 6.9 H, arrow).

6.2.5.2 Association between anti-Sall4 B immunostaining and histopathological parameters of prostate cancer in the Bath cohort

In order to evaluate if there was an association between anti-Sall4 B staining in normal vs. malignant prostate tissues, primary Gleason grades, clinical stages and biochemical relapse, nuclear anti-Sall4 B staining was quantified using the H-score system and compared to the histopathological and clinical features of PCa using the clinical data available for the Bath cohort. The range of nuclear anti-Sall4 B H-score in the Bath cohort varied between 0 - 100 (Figure 6.10). This analysis was carried out later in the project and the number of samples still available from the Bath cohort was reduced (14 PCa vs. 3 NP), for this reason, statistical tests were not carried out.

Quantification of the IHC showed increased nuclear anti-Sall4 B scores in PCa compared to NP tissues (Figure 6.10 A). There was a negative association between nuclear anti-Sall4 B staining and primary Gleason grade. Four of five (80%) PCa tissues with a low Gleason grade (3) had positive nuclear anti-Sall4 B staining, whereas, more than half of primary Gleason grade 4 tissues showed negative anti-Sall4 B immunostaining (Figure 6.10 B and Table 6.4). Nuclear anti-Sall4 B staining was not associated with clinical stage T (T1-2 vs. T3-4) (Table 6.4). There was no biological difference in the nuclear anti-Sall4 B staining between recurrent and non- recurrent PCa tissues (Table 6.4).

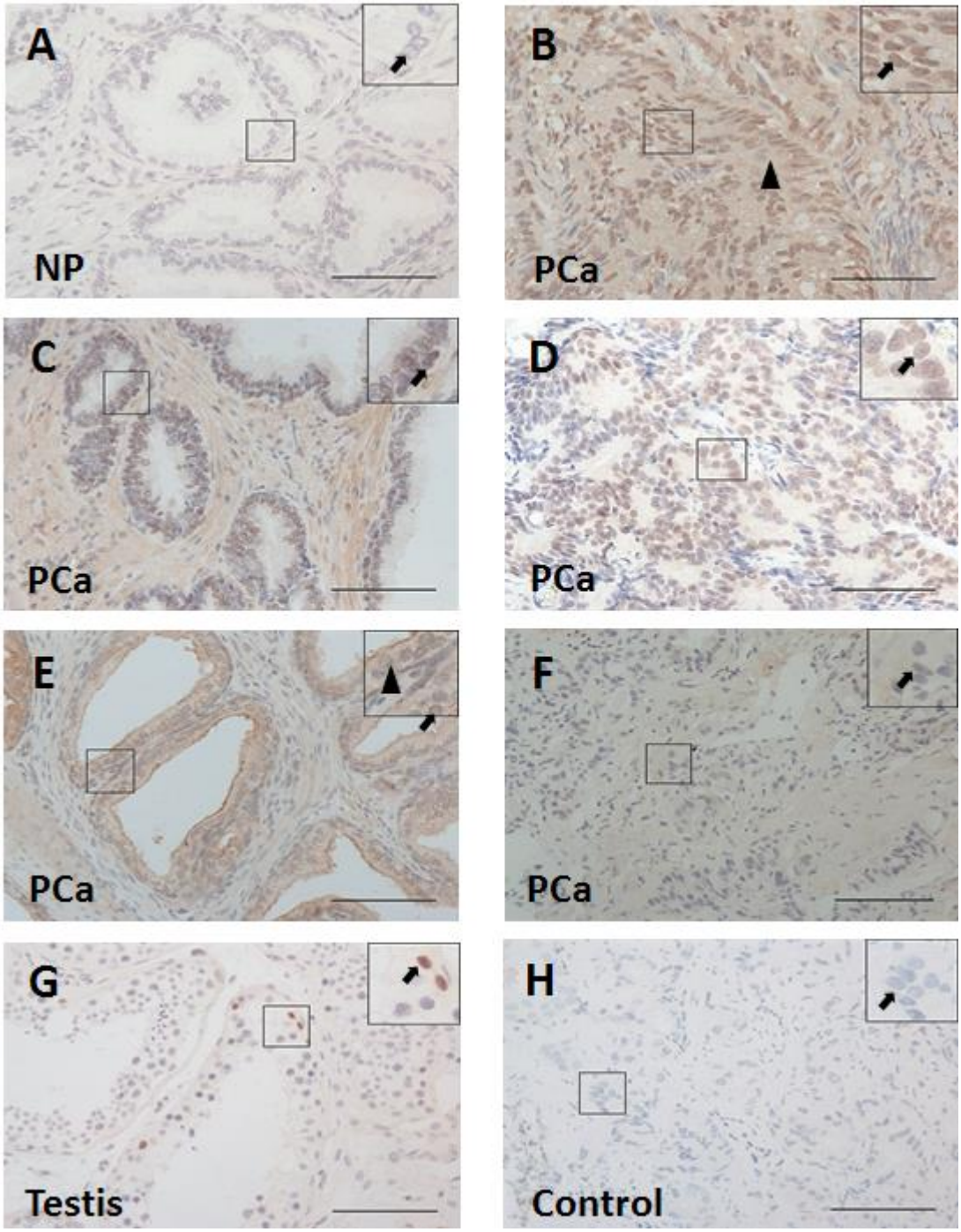

Figure 6. 9 Sall4 B expressions in samples from the Bath cohort, using anti-Sall4 antibody. Anti-Sall4 B was expressed heterogeneously in both normal and malignant tissues of the prostate. (A) NP had no anti-Sall4 B immunostaining (Black arrow). (B) Strong nuclear (Black arrow) and weak cytoplasmic (Black arrowhead) anti-Sall4 B staining in PCa. (C) Strong nuclear (Black arrow) anti-Sall4 B staining in PCa. (D) Moderate nuclear (Black arrow) Sall4 B staining in PCa. (E) Weak nuclear (Black arrow) and cytoplasmic (Black arrowhead) anti-Sall4 B staining in PCa. (F) Weak nuclear (Black arrow) anti-Sall4 B staining in PCa. (F) Weak nuclear anti-Sall4 staining (Black arrow) in PCa. (H) Strong nuclear anti-Sall4 B staining (Black arrow) in spermatogonia cells of normal testis tissues. (H) Negative control (no primary antibody added) showed free from background staining (Black arrow) in PCa. Scale bars=100µm with inserts at 2x zoom.

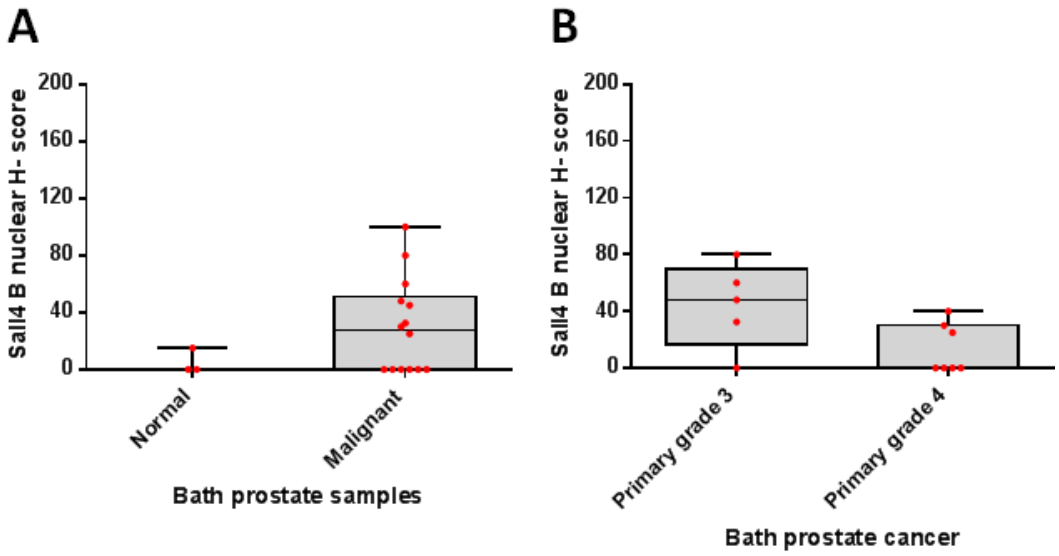

Figure 6. 10 Quantification of anti-Sall4 B nuclear staining in both normal and malignant Bath prostate tissues. Immunohistochemical staining of anti-Sall4 B was quantified in the Bath cohort using the H-score system for nuclear IHC staining. (A) Nuclear anti-Sall4 A staining was increased in PCa compared to NP tissues. (B) Nuclear anti-Sall4 B staining was reduced in primary Gleason grade 4 tissues compared to grade 3 tissues. Data represent the mean of five random images per case. NP (n=3) PCa (n=14), grade 3 (n=5), grade 4(n=7).

Table 6. 4 Nuclear anti-Sall4 B staining results with the Bath clinical data.

Comparison	Nuclear anti-Sall4 B staining
Normal vs malignant	Higher in malignant
Primary Gleason grades (3 & 4)	Lower in high grade
Stage (T) (T1-2vs.T3-4)	No biological difference
5 years Biochemical recurrence	No biological difference

In summary, nuclear anti-Sall4 B staining was increased in PCa compared to NP tissues and was negatively associated with increasing primary Gleason grade, but not with clinical stage T and biochemical recurrence. IHC Sall4 A&B data in normal vs. malignant prostate tissues was contradictory, whereas, the other association data was similar. The staining was repeated using the tissue microarray with a larger sample size.

6.2.6 Expression of Sall4 B in the TMA cohort, using the anti-Sall4 B antibody

IHC was carried out on prostate tissue samples from the TMA cohort, using the anti-Sall4 B antibody.

6.2.6.1 Anti-Sall4 B staining in normal and malignant TMA prostate samples

IHC showed that nuclear anti-Sall4 B staining was not detected in NP tissues (Figure 6.11 A, arrow). In contrast, nuclear anti-Sall4 B staining was observed in PCa tissues and the intensity of signal varied widely, from strong and widespread (Figure 6.11 B&C, arrows), moderate (Figure 6.11 D&E, arrows), weak (Figure 6.11 F, arrow) and negative (Figure 6.11 G, arrow). A negative control showed no significant background staining in prostate tissue (Figure 6.11 H, arrow).

6.2.6.2 Association between anti-Sall4 B staining and histopathological parameters of prostate cancer from the TMA cohort

Having carried out IHC staining, the nuclear anti-Sall4 B staining was quantified using H-score system as carried out for the Bath cohort samples and then compared to normal vs malignant prostate tissues and other histopathological parameters, including primary Gleason grade, clinical stage and biochemical recurrence. The range of nuclear anti-Sall4 B H-score in the TMA varied between 0-80 (Figure 6.12).

The first analysis looked at the nuclear anti-Sall4 B staining in normal vs. malignant prostate tissues. Quantification of the nuclear anti-Sall4 B showed increased nuclear anti-Sall4 B staining significantly in PCa tissues compared to NP tissues from the TMA cohort ($p= 0.0456$) (Figure 6.12 A & Table 6.5). There was a significant difference in the mean of nuclear anti-Sall4 B scores among primary Gleason grades ($p= <0.0001$), using an ANOVA test (Figure 6.12 B & Table 6.5). Detailed analysis of the IHC data, using multi-comparison (Tukey) tests showed nuclear anti-Sall4 B staining negatively associated with increasing primary Gleason grades. Nuclear anti-Sall4 B staining was reduced significantly when comparing PCa patients with a primary Gleason grade 3 to those with a primary Gleason grade 4 ($p=0.0002$) or a grade 5 ($p=<0.0001$) (Figure 6.12 B & Table 6.5). There was no significant difference in mean of the nuclear anti-Sall4 B score between primary Gleason grade 4 and grade 5 tissues ($p= 0.2657$) (Figure 6.12 B & Table 6.5). In contrast, there was no significant association between nuclear anti-Sall4 B staining and clinical stage TNM (Table 6.5). This result was agreed with anti-Sall4 B result in the Bath cohorts.

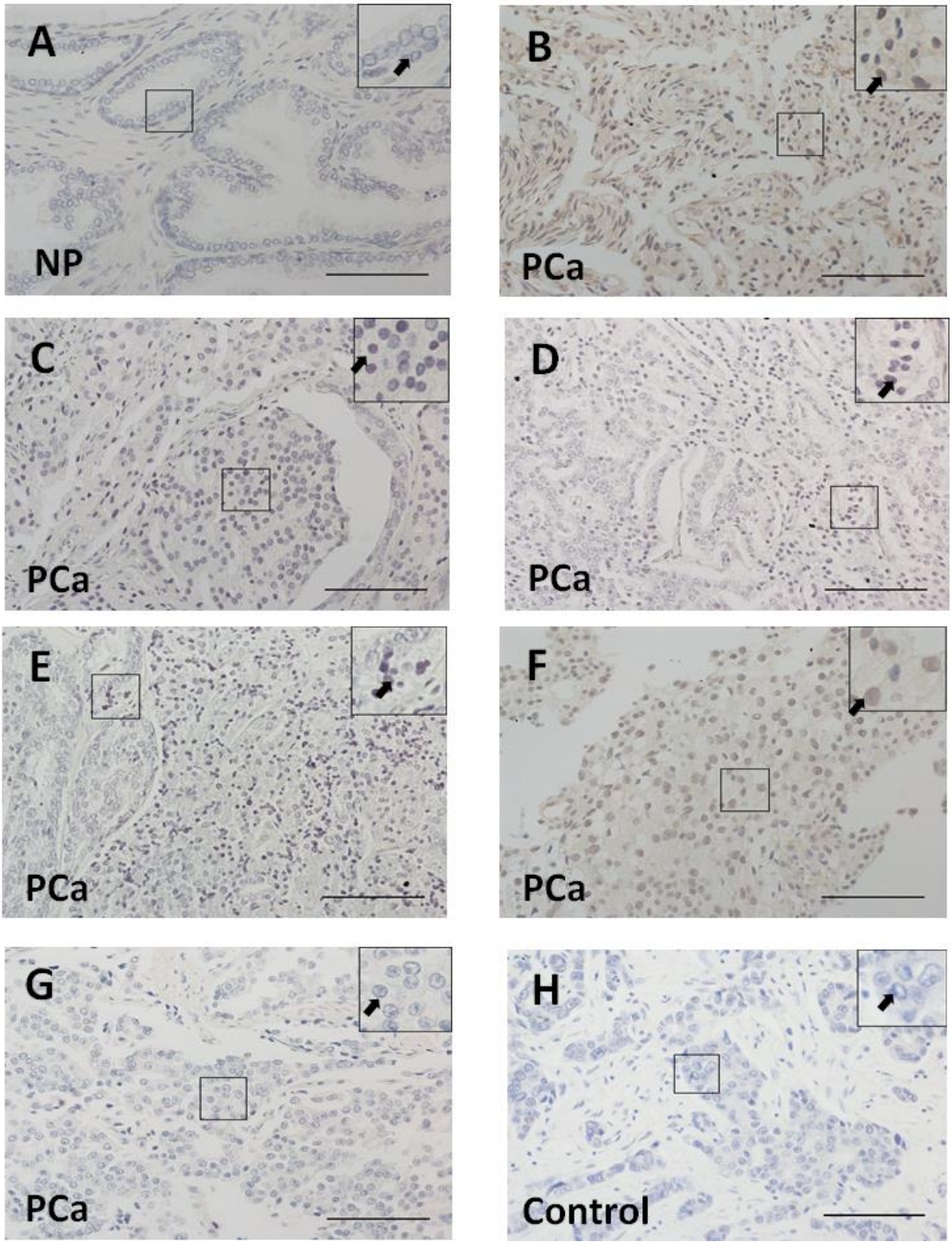

Figure 6. 11 Sall4 B expressions in samples from the TMA cohort, using anti-Sall4 B antibody. Anti-Sall4 B was expressed heterogeneously in both normal and malignant tissues of the prostate. (A) Negative anti-Sall4 B staining (Black arrow) in NP. (B) Strong nuclear (Black arrow) anti-Sall4 B staining in PCa. (C) Strong nuclear anti-Sall4 B staining (Black arrow) in PCa. (D) Moderate nuclear and widespread anti-Sall4 B staining (Black arrow) in PCa. (E) Moderate nuclear (Black arrow) anti- Sall4 B staining in PCa. (F) Weak nuclear anti-Sall4 B staining (Black arrow) in PCa. (G) PCa had strong staining for anti-Sall4 B (Black arrow). (H) Negative control (no primary antibody added) showed free from background staining (Black arrow) in PCa. Scale bars=100µm with inserts at 2x zoom.

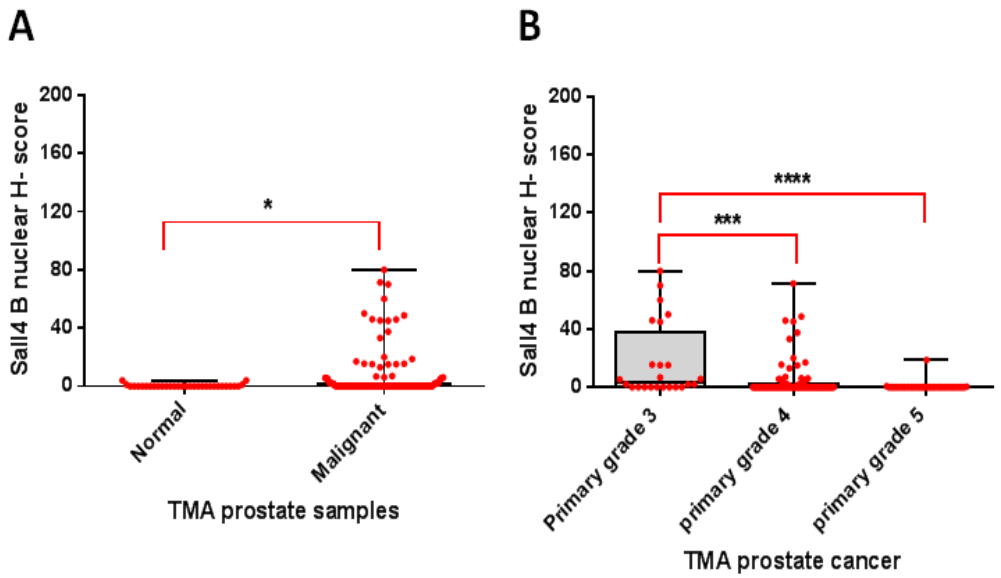

Figure 6. 12 Quantification of anti-Sall4 B nuclear staining in both normal and malignant TMA prostate tissues. Immunohistochemical staining of anti-Sall4 B was quantified in the TMA cohort using H-score system for nuclear IHC staining. (A) Nuclear anti-Sall4 B staining was significantly increased in PCa compared with NP (p=0.0456). (B) Nuclear anti-Sall4 B staining showed a significant difference among the primary Gleason grades, using An ANOVA test (p=< 0.0001). Multi-comparison (Tukey) tests showed a significant reduction in the nuclear anti-Sall4 B score when comparing grade 3 to grade 4 (p=0.0002) or grade 5 tissues (p=<0.0001). There was no significant difference in the mean of nuclear anti-Sall4 B staining between primary Gleason 5 and grade 4 (p=0.3657). Data represent the mean of five random images per case. Unpaired or one-way ANOVA tests were conducted to determine the statistical difference for each set of conditions. NP (n=16), PCa (80), grade 3 (n=13), grade 4 (n=46) and grade 5 (n=18).

Table 6. 5 Nuclear anti-Sall4 B staining results with the TMA clinical data.

Comparison	**Nuclear anti- Sall4 B staining**		
	Results		**p. value**
Normal vs malignant	Higher in malignant		0.0456
Primary Gleason grades (3, 4 &5).	Lower in high Gleason grade	Anova test	<0.0001
		Grade 4 vs. Grade 3	0.0002
		Grade 5 vs. Grade 3	<0.0001
		Grade 5 vs. Grade 4	0.2657
Stage (T)	No statistically significant difference		0.5045
Stage (M)	No statistically significant difference		0.5411
Stage (N)	No statistically significant difference		0.0899

In summary, nuclear anti-Sall4 B staining was increased significantly in PCa compared to NP and was negatively associated with increasing primary Gleason grade, but not with the clinical stage (TNM). These results agreed with anti-Sall4 B results in the Bath cohort results. However, they only partially agreed with the Sall4 A results from both cohorts, where the Sall4 A antibody showed decreased staining in the malignant prostate.

6.2.7 Analysis of expression of Sall4 mRNA using RNAscope®

The IHC results of anti-Sall4 A&B staining between normal and malignant prostate tissue samples were contradictory, with one antibody showing an increase and the other a decrease in expression in malignant tissues. Measuring Sall4 mRNA expression using RNAscope® (Described in Chapter 6.1.2) provided an alternative way of trying to establish which pattern was correct, so Sall4 mRNA levels were investigated on normal and malignant prostate tissues from the Bath and TMA cohorts using the RNAscope® assay.

6.2.7.1 Testing the RNAscope® protocol

In order to test if the RNAscope® protocol was working correctly, it was performed with positive (PPIB, *Homo sapiens* peptidylprolyl isomerase B (cyclophilin B), mRNA) and negative (Dap B, a bacterial gene) controls on HeLa cells and prostate tissue samples.

The positive control probe (PPIB) showed strong brown punctate dots located in the nuclei and cytoplasm of HeLa cells (Figure 6.13 A) and prostate tissue (Figure 6.12 C). In contrast, the negative control probe (Dap B) showed no clear background staining in both HeLa cells (Figure 6.13 B) and prostate tissue (Figure 6.13 D). This confirmed that the RNAscope® was working as expected and so the technique was then used to investigate Sall4 mRNA expression in prostate samples.

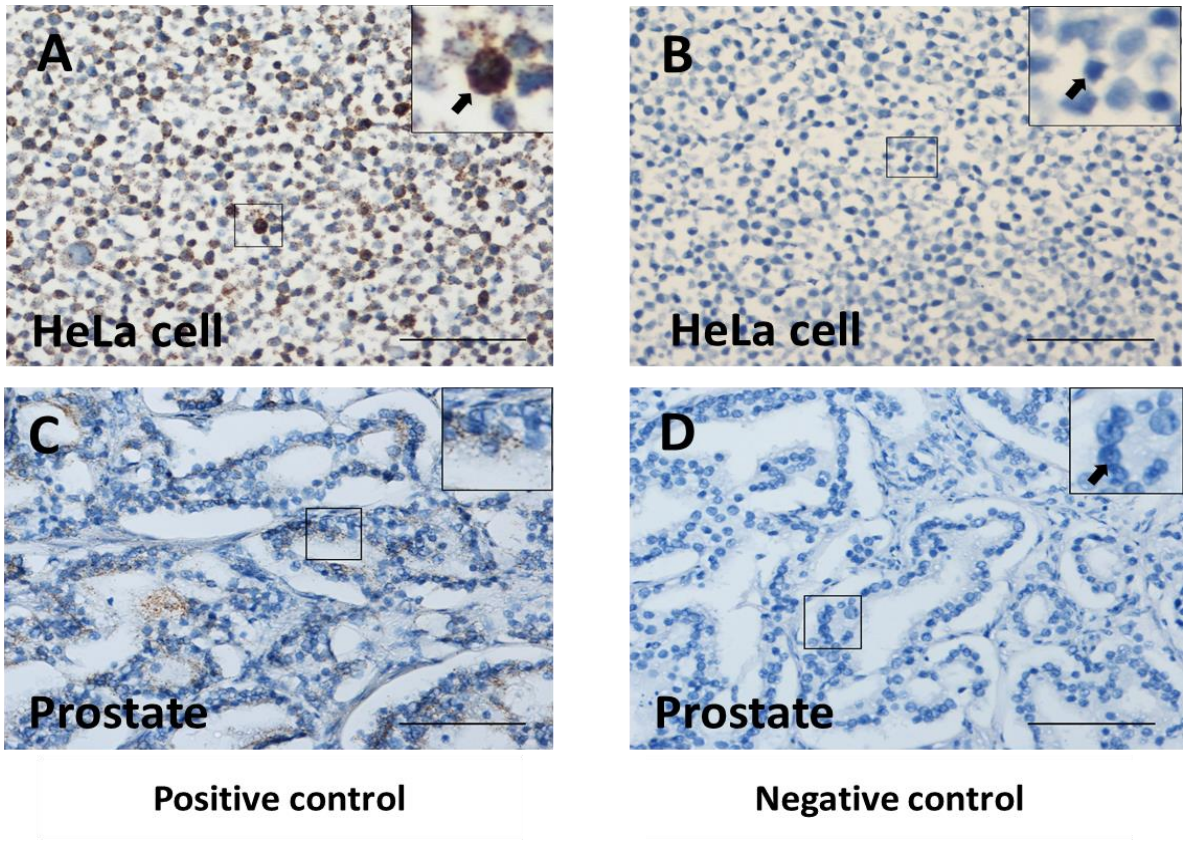

Figure 6. 13 RNA scope: Positive and negative control expression in HeLa cells and prostate tissue. (A&C) Strong nuclear and cytoplasmic PPIB staining in HeLa cells and prostate tissue. (B&D) Dap B showed no clear background staining in HeLa cells and prostate tissue. The presence of mRNA is shown in brown dots. Blue staining is a hematoxylin counterstain. Scale bars=100µm with inserts at 2x zoom.

6.2.7.2 Sall4 mRNA expression in prostate tissues from the Bath cohort

RNAscope® analysis was then carried out on prostate tissue samples from the Bath cohort. The result showed that Sall4 mRNA staining was observed in normal and malignant prostate tissue samples (Figure 6.14). A quarter of NP tissues (1/4) had Sall4 mRNA staining in the nuclei and/ or cytoplasm of glandular epithelial cells (Figure 6.14 A, arrow), whereas three quarters (75%) of them had no Sall4 mRNA staining (Figure 6.14 B, arrow). Sall4 mRNA staining was also found in PCa tissues and the number of positive cells varied widely, from a widespread (Figure 6.14 C&D, arrows), scattered (Figure 6.14 E, arrow) and negative (Figure 6.14 F, arrow). In testis tissue, Sall4 mRNA was expressed in spermatogonia cells (Figure 6.14 G, arrow). A negative control showed no detectable background staining in prostate tissue (Figure 6.14 H, arrow).

6.2.7.3 Association between Sall4 mRNA staining and histopathological parameters of prostate cancer tissues from the Bath cohort

Histologically normal and malignant prostate tissues were stained for Sall4 mRNA and scored using the Sall4 mRNA scoring system (Described in chapter 2.2.3). The range of Sall4 mRNA score in the TMA varied between 0-3.6 (Figure 6.15). This analysis was carried out later in the study and the number of samples still available from the Bath cohort was reduced (12 PCa vs. 4 NP), for this reason, statistical tests were not carried out.

The RNAscope® staining showed increased Sall4 mRNA staining in PCa tissue samples compared to NP tissues. A quarter (1/4) of the NP tissues showed positive staining for Sall4 mRNA, whereas, 50% (6/12) of PCa had a positive Sall4 mRNA staining (Figure 6.15 A). There was also a reduction in the staining of Sall4 mRNA with increasing primary Gleason grade. 75% of PCa with grade 3 (3/4 cases) has a positive Sall4 mRNA staining, whereas, the percentage of positive Sall4 mRNA staining was reduced to 33% in PCa with a primary Gleason grade 4 (2/6 cases) (Figure 6.15 B and Table 6.5). Sall4 mRNA staining in localised PCa T1-2 was higher than advanced stage T3-4 (Figure 6.15 C& Table 6.6) and there was a negative association between Sall4 mRNA staining and biochemical recurrence. 75% of non relapsed PCa had positive staining for Sall4 mRNA, whereas, 100% of relapsed PCa showed negative staining for Sall4 mRNA (Figure 6.15 D& Table 6.6).

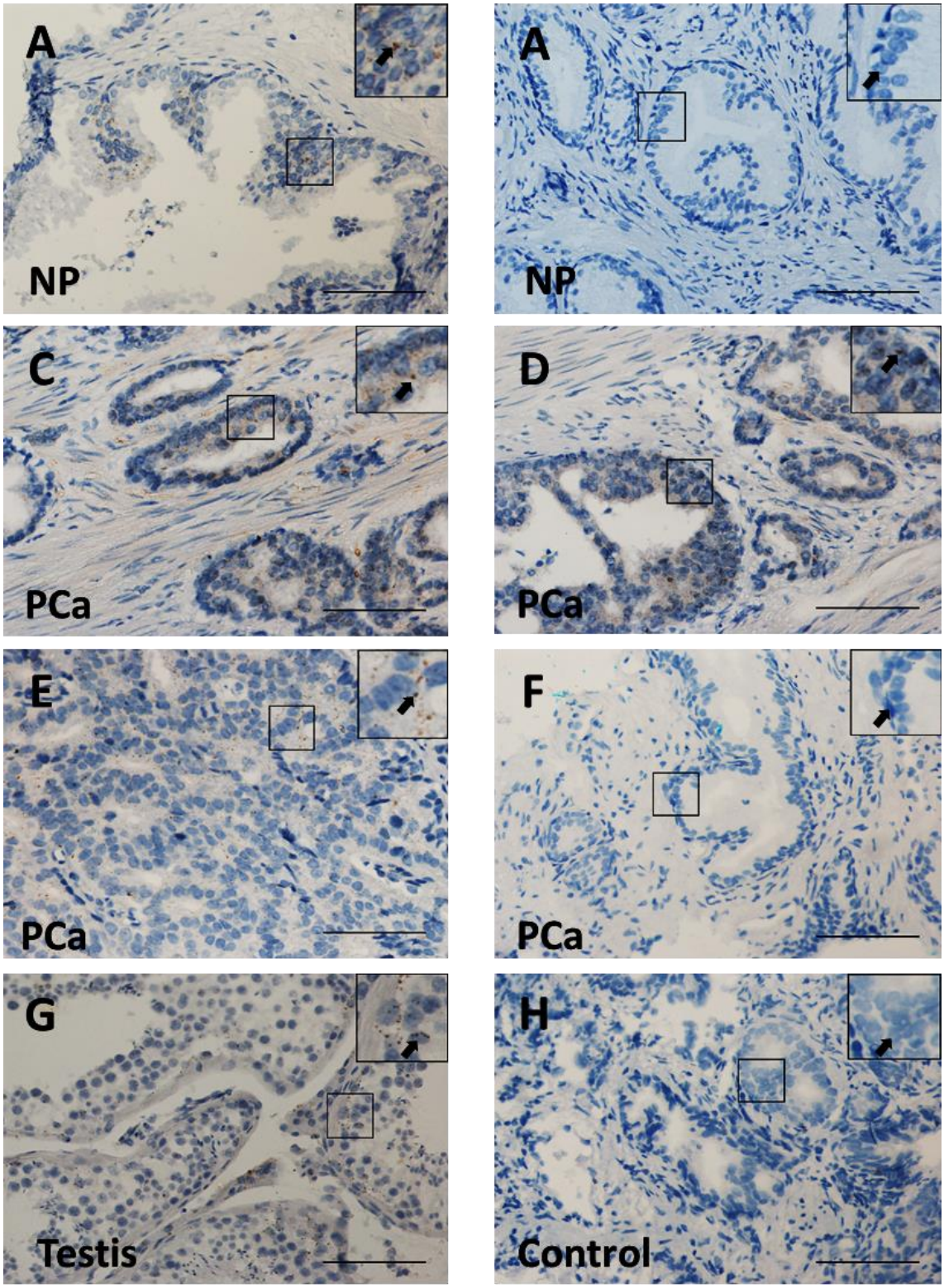

Figure 6. 14 Sall4 mRNA staining in prostate samples from the Bath cohort. (A) Sall4 mRNA staining (Black arrow) in some NP epithelial cells (B) No Sall4 mRNA staining in NP tissue (Black arrow). (C) Widespread Sall4 mRNA staining (Black arrow) in PCa tissues. (D) Widespread Sall4 mRNA staining (Black arrow) in PCa tissues. (E) scattered Sall4 mRNA staining (Black arrow) in PCa. (F) No positive staining for Sall4 mRNA (Black arrow) in PCa. (G) Spermatogonia cells of normal testis tissues had a positive Sall4 mRNA staining (Black arrow). (H) Negative control showed a free background staining (Black arrow) in PCa. Scale bars=100µm with inserts at 2x zoom

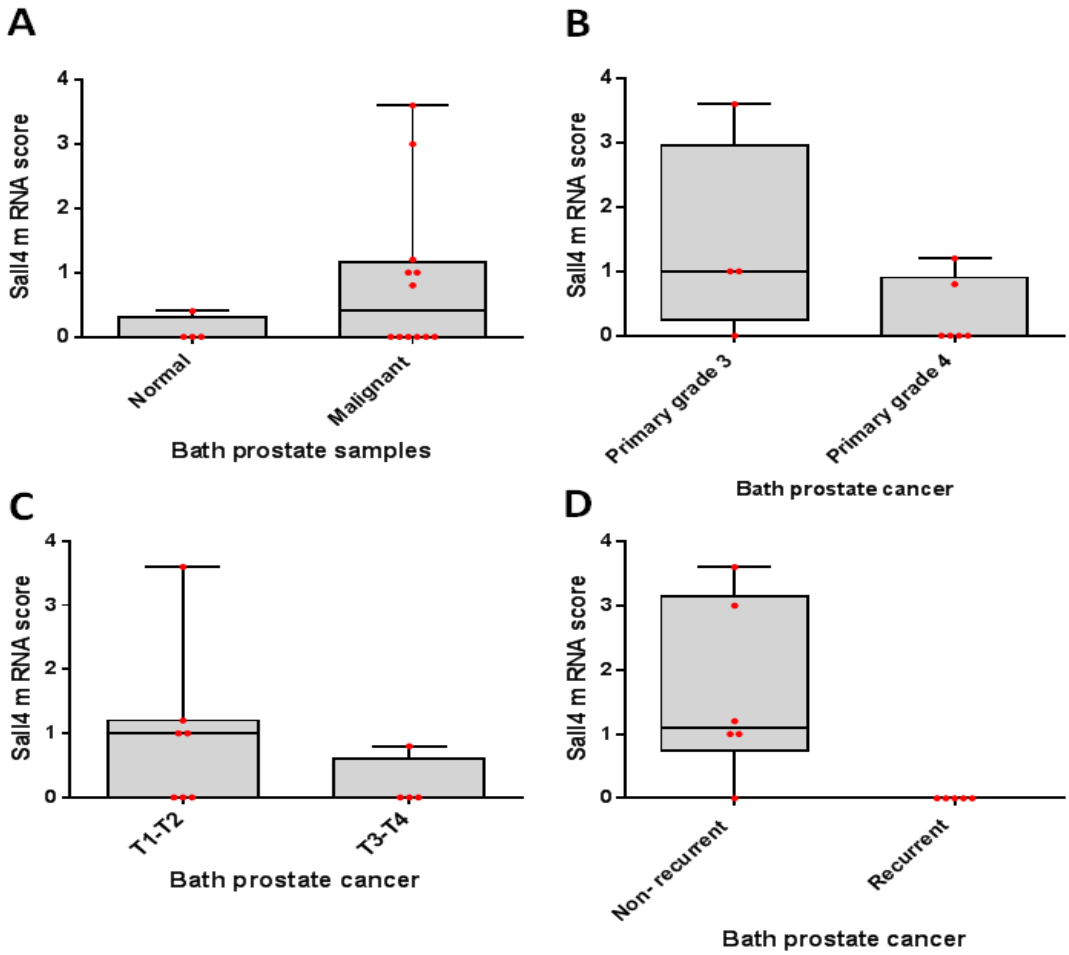

Figure 6. 15 Quantification of Sall4 mRNA staining in both normal and malignant Bath prostate tissues. RNAscope® staining of Sall4 mRNA was quantified in the Bath cohort using Sall4 mRNA score. (A) Sall4 mRNA staining was increased in PCa compared with NP tissues (B) Sall4 mRNA staining was negatively associated with increasing primary Gleason grade. (C) Sall4 mRNA staining was not associated with the clinical stage (T3-4). (D) Sall4 mRNA staining was negatively associated with a biochemical recurrence. Data represent the mean of five random images per case. NP (n=4), PCa (12), grade 3 (n=4), grade 4 (n=6), T1-2 (n=7), T3-4(n=4), non-recurrent (n=6) and recurrent (n=4). Due to the small sample size, no statistical tests were used.

Table 6. 6 Summary of m RNA Sall4 result with clinical data of the Bath cohort

Comparison	Sall4 mRNA staining
Normal vs malignant	Higher in malignant
Primary Gleason grades (3 & 4)	Lower in high grade
Clinical Stage (T)	No biological difference
5 years Biochemical recurrence	Lower in biochemical recurrence

In summary, Sall4 mRNA staining was increased in PCa and was negatively associated with increasing grade and biochemical relapse. This result largely agreed with the anti-Sall4 B results and also partially agreed with anti-Sall4 A staining in both cohorts. However, the data is based on a small cohort so RNAscope® was used to measure the expression of Sall4 in the larger TMA cohort.

6.2.8 Sall4 mRNA expression in prostate tissues from the TMA cohort

RNAscope® was carried out on prostate tissue samples from the TMA cohort.

6.2.8.1. Sall4 mRNA staining in normal vs malignant prostate tissue from the TMA cohort

NP tissues showed no significant staining for Sall4 mRNA (Figure 6.15 A, arrow). In contrast, Sall4 mRNA staining was detected in PCa tissues and the number of positive cells varied widely, from widespread (Figure 6.16 B, arrow), scattered (Figure 6.16 C&D, arrows) and negative (Figure 6.16 E, arrow). A negative control was free from background staining in prostate tissue (Figure 6.16 F, arrow). This result was consistent with Sall4 protein and mRNA results in both cohorts.

6.2.8.2 Association between Sall4 mRNA staining and histopathological parameters of prostate cancer from the TMA cohorts

In order to determine if there an association between Sall4 mRNA staining in normal vs. malignant prostate tissues and/or different primary Gleason grades, clinical stages and biochemical relapse, Sall4 mRNA staining was assessed using Sall4 mRNA score and then compared to histopathological features of PCa using the clinical data available for the TMA cohort.

The first analysis looked at Sall4 mRNA staining in normal and malignant prostate tissues from the TMA cohort. Quantification of RNAscope® showed that Sall4 mRNA staining was significantly increased in PCa compared to NP tissues (p= 0.0007) (Figure 6.17 A & Table 6.7). There was also a significant difference in the mean of Sall4 mRNA scores among primary Gleason grades (p= 0.0109), using an ANOVA test (Figure 6.17 B & Table 6.7). Sall4 mRNA staining was significantly reduced when comparing PCa patient with primary Gleason grade 3 tissues to those with grade 4 (p=0.0499) or grade 5 tissues (p=0.0081) (Figure 6.17 B & Table 6.7). There was no significant difference in the mean of Sall4 mRNA score between primary Gleason grade 4 and grade 5 (p= 0.4070) (Figure 6.17 B & Table 6.7). In contrast, Sall4 mRNA staining was not associated significantly with clinical stage TNM (Table 6.7).

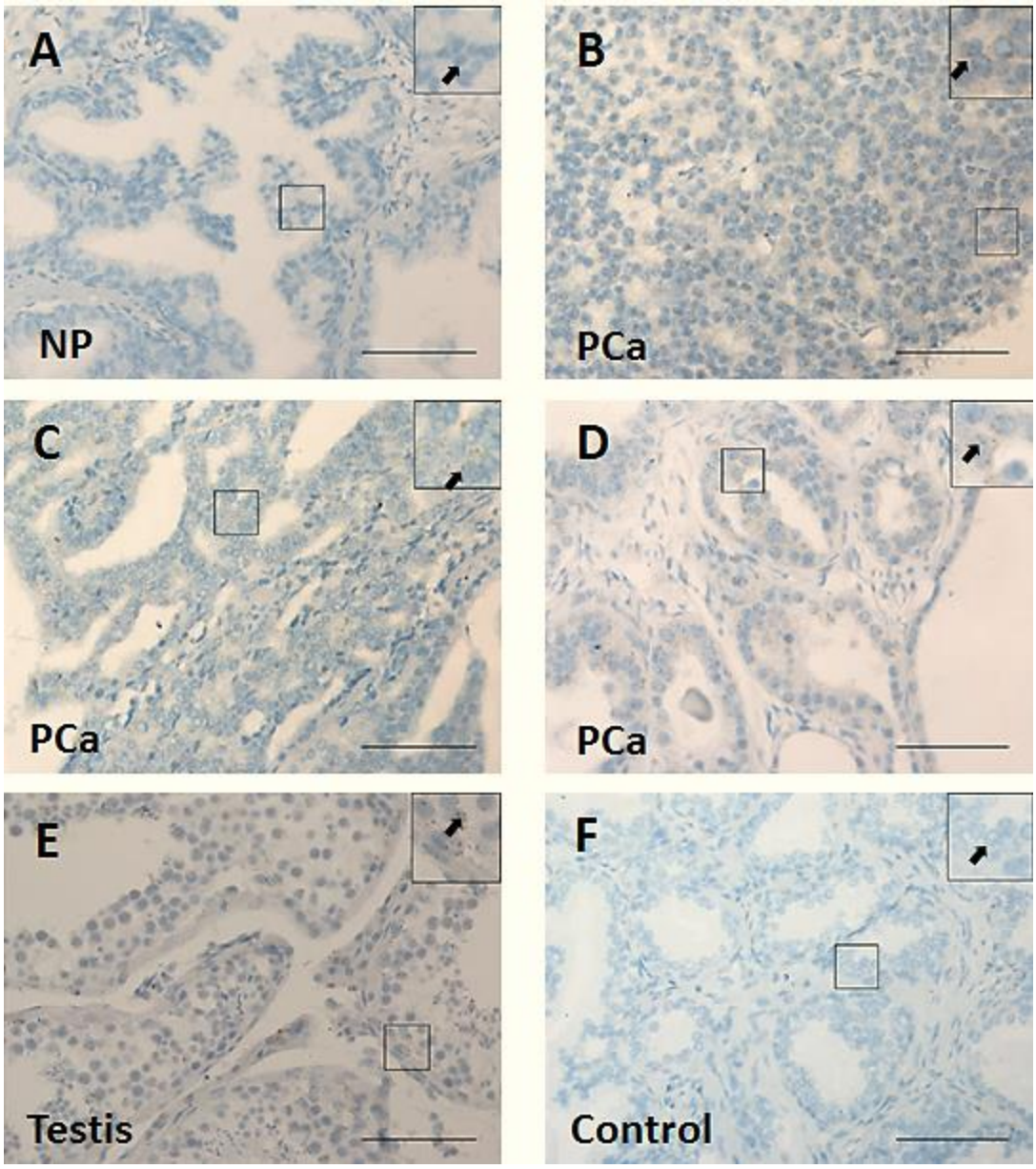

Figure 6. 16 Sall4 mRNA expression in prostate samples from the TMA cohort. (A) Negative staining for Sall4 mRNA (Black arrow) in NP. (B) Widespread Sall4 mRNA (Black arrow) in PCa. (C) Scattered Sall4 mRNA staining (Black arrow) in PCa. (D) Scattered Sall4 mRNA staining (Black arrow) in PCa. (E) Negative staining for Sall4 mRNA (Black arrow) in PCa. (F) The negative control was free from background staining (Black arrow) in PCa. Scale bars=100µm with inserts at 2x zoom.

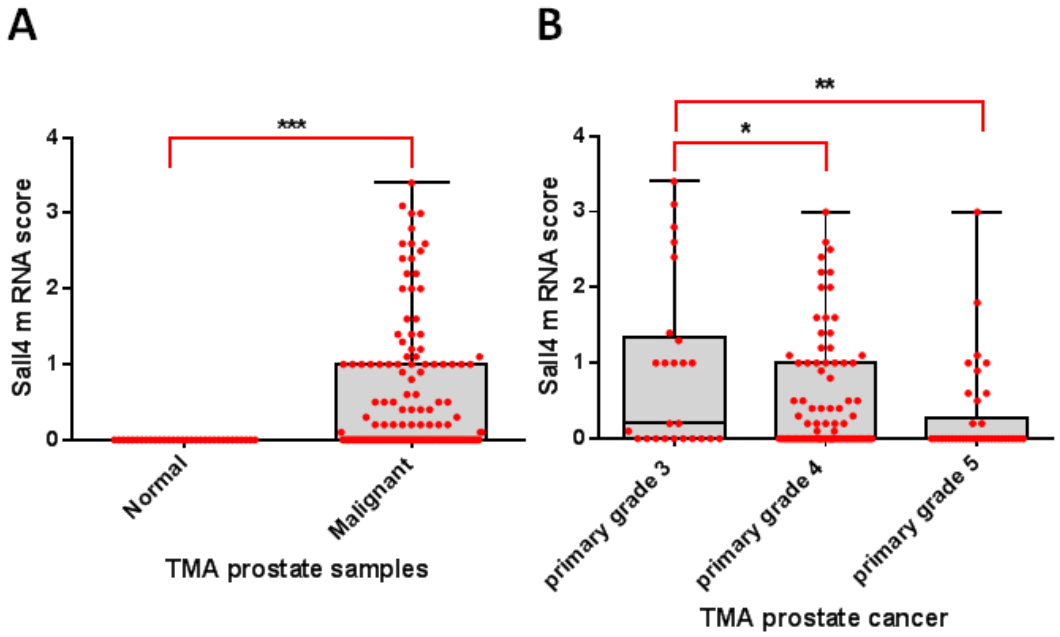

Figure 6. 17 Quantification of Sall4 mRNA staining in both normal and malignant TMA prostate tissues. Sall4 mRNA staining was quantified in the TMA cohort using Sall4 mRNA score. (A) Sall4 mRNA staining was significantly increased in PCa compared with NP tissues (p=0.0007). (B) Sall4 mRNA staining showed a significant difference among primary Gleason grades, using An ANOVA test (p=0109). Multi comparison (Tukey) tests showed a significant reduction in Sall4 mRNA staining when comparing grade 3 tissues to those with a grade 4 (p=0.0499) or grade 5 (p=0.0081). Sall4 mRNA staining was also reduced in Gleason 5 compared to Grade 4 but this reduction was not significant (p=0.3657). Data represent the mean of five random images per case. Unpaired T-test or one-way ANOVA tests were conducted to determine the statistical difference for each set of condition. NP (n=16), PCa (n=80), grade 3 (n=13), grade 4 (n=46) and grade 5 (n=18).

Table 6. 7 Sall4 mRNA staining results with the TMA clinical data.

Comparison	**Sall4 mRNA staining**		
	Results		**p. value**
Normal vs malignant	Higher in malignant		0.0007
Primary Gleason grades (3, 4 &5).	Lower in high Gleason grade	Anova test	0.0109
		Grade 4 vs. Grade 3	0.0499
		Grade 5 vs. Grade 3	0.0081
		Grade 5 vs. Grade 4	0.4070
Stage (T)	No statistically significant difference		0.6781
Stage (M)	No statistically significant difference		0.822
Stage (N)	No statistically significant difference		0.4469

In summary, Sall4 mRNA staining was increased significantly in PCa and was negatively associated with increasing primary Gleason grade in the TMA cohort. However, its expression was not associated with clinical stage. These results were consistent with those from anti-Sall4 B. They were also partially agreed with the anti-Sall4 A results, the main significant difference was that anti-Sall4A staining showed a reduction in PCa rather than an increase. The results from the two antibodies and the RNAscope with the two cohorts are

summarised below (Table 6.8). The Sall4 hypothesis in this study is accepted partially by IHC anti Sall4 B and mRNA probe.

6.2.9 Sall4 results in the Bath and TMA cohorts: testing the hypothesis

The results from the two cohorts and if the hypothesis is accepted or rejected is summarised in Table 5.8.

Table 6. 8 The summary of Sox7 results in the Bath and TMA cohorts

Parameters	Localisation	hypothesis	Bath	TMA	Results Confirmed?	Hypothesis Accepted?
Normal vs Cancer	Nuclear Sall4 A	Sall4 will be increased in PCa	Sall4 is decreased in PCa	Sall4 is decreased in PCa	Yes	NO
	Nuclear Sall4 B		Sall4 is increased in PCa	Sall4 is increased in PCa	Yes	Yes
	Sall4 mRNA		Sall4 is increased in PCa	Sall4 is increased in PCa	Yes	Yes
Primary Gleason grades	Nuclear Sall4 A	Sall4 will be not associated with grade	Sall4 is decreased in PCa	Sall4 is decreased in PCa	Yes	NO
	Nuclear Sall4 B		Sall4 is decreased in PCa	Sall4 is decreased in PCa	Yes	NO
	Sall4 mRNA		Sall4 is decreased in PCa	Sall4 is decreased in PCa	Yes	NO
Stage T	Nuclear Sall4 A	Sall4 will be not associated with stage	No significant difference	No significant difference	Yes	Yes
	Nuclear Sall4 B		No significant difference	No significant difference	Yes	Yes
	Sall4 mRNA		No significant difference	No significant difference	Yes	Yes
Stage M	Nuclear Sall4 A		Not tested	No significant difference	N/A	Yes
	Nuclear Sall4 B		Not tested	No significant difference	N/A	Yes
	Sall4 mRNA		Not tested	No significant difference	N/A	Yes
Stage N	Nuclear Sall4 A		Not tested	No significant difference	N/A	Yes
	Nuclear Sall4 B		Not tested	No significant difference	N/A	Yes

	Sall4 mRNA		Not tested	No significant difference	N/A	Yes
Relapsed Vs. Non-relapsed	Nuclear Sall4 A	Sall4 will be not associated with relapse	No significant difference	Not tested	N/A	Yes
	Nuclear Sall4 B		No significant difference	Not tested	N/A	Yes
	Sall4 mRNA		No significant difference	Not tested	N/A	Yes

6.3 Discussion

6.3.1. Summary

By examining Sall4 expression at protein and mRNA levels in normal and malignant prostate tissue samples from the Bath and TMA cohorts, using IHC and RNAscope® assays, respectively, the present study showed that Sall4 protein and mRNA staining was significantly increased in PCa compared to NP tissues from both cohorts, using anti-Sall4 B antibody and Sall4 mRNA probe. In contrast, nuclear anti- Sall4 A was reduced significantly in PCa compared to NP tissues from both cohorts. Sall4 protein and mRNA levels were negatively associated with increasing primary Gleason grade in both cohorts using two different anti-Sall4 antibodies (A&B) and Sall4 mRNA probe. In contrast, Sall4 protein and mRNA level did not show a clear association with the clinical stage (TNM) and the risk of biochemical recurrent in both cohorts. In this chapter, I will consider how this pattern of expression compares to previous studies, the differences between IHC and RNAscope® for biomarker analysis and the possible role of Sall4 in PCa.

Table 6. 9 Confirmed patterns of expression for Sall4.

Biomarker	Confirmed patterns of expression
Anti-Sall4 A	Anti-Sall4 A staining was reduced significantly in PCa and was negatively associated with increasing primary Gleason grade in the Bath and TMA cohorts.
Anti-Sall4 B	Anti-Sall4 B staining was increased in PCa and was negatively associated with increasing primary Gleason grade in the Bath and TMA cohorts.
Sall4 mRNA	Sall4 mRNA staining was increased in PCa and was negatively associated with increasing primary Gleason grade in the Bath and TMA cohorts.

6.3.2 Sall4 is increased significantly in prostate cancer and is negatively associated with increasing primary Gleason grade but not clinical stage and biochemical relapse

This study showed two approaches that Sall4 staining was significantly increased in PCa compared to NP tissues. Therefore, it appears most likely that Sall4 expression is increased in PCa compared to NP tissues. However, given the contradictory result with the Sall4 A antibody, future work should confirm this finding.

To the best of my knowledge, Sall4 staining in normal vs. malignant prostate tissues and the association between expression and primary Gleason grade, clinical stage and biochemical relapse has not been previously examined and this study is the first to investigate if there is an association between Sall4 and clinical parameters in PCa. However, there are three previous studies, focusing on other cancer tissues, that have shown nuclear Sall4 staining in normal or malignant prostate tissues. These reports have not determined the association between nuclear Sall4 expression in normal vs. malignant prostate tissues or other PCa clinical features. For example, a study on non- small cell lung carcinomas used a TMA with 112 normal tissues as controls, including 7 NP tissues and showed weak nuclear Sall4 staining in 57% of NP tissues (4/7) (Rodriguez *et al.*, 2014). This result was consistent with the Bath cohort data and anti-Sall4 A data in the TMA cohort. Another IHC study with a large TMA of human cancer tissues, including 62 PCa showed no nuclear Sall4 staining in all tumour tissues of the prostate (Kilic *et al.*, 2016). This result was of some consistency with our results in poorly differentiated PCa tissues and because the authors did not mention the clinical data of PCa sample and these samples might be poorly differentiated. In addition, a third study was on metastatic germ & non-germ cell tumours and showed all metastatic PCa tissue samples (12 cases only) had no Sall4 staining (Cao *et al.*, 2009). The work presented here did not look at metastatic samples, but high grade tumours did show lower levels of Sall4 which is consistent with a model where more advanced tumours such as high grade, or metastatic tumours, have lower expression of Sall4. The data in this thesis agrees with some previous studies looking at Sall4 in other types of cancers, including lung and colon carcinomas (Forghanifard *et al.*, 2013; Rodriguez *et al.*, 2014). However, the anti-Sall4 B and mRNA data presented here did not agree with the study done by Hao, *et al.* that showed a reduction Sall4 staining in colorectal carcinoma compared to atypical hyperplasia or normal colon tissues (Hao *et al.*, 2016). However, this study was consistent with the anti-Sall4 A data. Hao *et al* used a mouse monoclonal antibody but we believe that this antibody

is different from our antibodies. The complicated and partially contradictory IHC results for nuclear Sall4 staining in normal vs. malignant tissues from different cancers suggests Sall4 expression might show increased and decreased expression depending on the cancer type. However, different studies have also used different antibodies and different concentrations that might affect IHC results. Further work needs to be carried out using tissue from large cohorts of different cancer types and measuring the expression of both mRNA and protein to establish if differences between studies are due to differences between tumour type or the reagents used.

In term of an association between Sall4 staining with primary Gleason grade, the current study showed that Sall4 staining was negatively associated with increasing primary Gleason grades in both cohorts, using two different antibodies and mRNA probes for Sall4. This result was consistent with the previous studies on colorectal carcinoma (Hao *et al.*, 2016) and suggests that Sall4 could play a role in promoting cell differentiation in lower grade PCa and might be a potential biomarker for PCa prognosis. In contrast, another study showed Sall4 mRNA levels in moderate differentiated colorectal cancer tissues were lower than well and poorly differentiated tissues, using RT-PCR (Forghanifard *et al.*, 2013). This study, however, was on colorectal carcinoma and used RT-PCR as a different diagnostic method for measuring Sall4 mRNA. Other reports showed there was no significant association between Sall4 expression and grades of cancers, including lung and hepatocellular carcinoma (Han *et al.*, 2014; Rodriguez *et al.*, 2014; Gautam *et al.*, 2015). These studies used different cancer types, suggesting that there may be multiple roles for Sall4 in cancer depending on cancer types.

Our data showed no significant association between Sall4 staining and clinical stage in both cohorts, suggesting Sall4 expression does not link to PCa clinical stage (TNM). The results were consistent with the previous findings in other cancers, including lung cancer (Gautam *et al.*, 2015; Rodriguez *et al.*, 2014), oesophagus (Kilic *et al.*, 2016) and hepatocellular carcinomas (Han *et al.*, 2014). In colorectal carcinoma, Sall4 mRNA expression was also not associated with clinical stage T, but was positively associated with a tumour metastatic to lymph nodes (Forghanifard *et al.*, 2013). This could be caused by a difference in tumour type, however, it used a small sample size divided into three groups, including N0: 28, N1:9 & N2:3. A three-group statistical analysis with this small sample size may not provide a reliable guide to show if there is a real biological difference.

Finally, the present study suggests that there was no significant association between Sall4 protein and mRNA staining and a risk of biochemical relapse in the Bath cohort. There was no previous study in PCa, but the present study did not agree with the previous study on hepatocellular carcinoma that showed increased Sall4 protein in serum of the hepatocellular carcinoma relapsed patients compared with non- relapsed, using RT-PCR assay (Han *et al.*, 2014). The study, however, used the serum samples from hepatocellular carcinoma patients and also used a different method of detection. There are also still many questions about our data because the Bath prostate sample size was small and the TMA cohort lacked clinical data regarding relapse and non-relapse status. It would be very interesting to assess the expression of Sall4 at protein and mRNA level, using IHC and RNAscope in a large TMA cohort which has clinical information about a risk of biochemical relapse (see Chapter 8 for more details).

6.3.3 RNAscope vs IHC staining for biomarker analysis

Different methods are used for PCa diagnosis and prognosis in the clinic, including serum PSA level, Gleason score and clinical stage, as well as the identification of tumour markers using IHC. Immunohistochemistry represents one of these widely used methods in clinical practice and identifies several components of cells and tissues, including proteins (Oliver and Jamur, 2010; Wang *et al.*, 2012). However, IHC results can be affected by many factors. Using different antibodies, different antibody concentrations or antigen retrieval methods may show different IHC results (Whitaker *et al.*, 2008). The absence of a standard protocol for IHC represents another disadvantage from using IHC. Finally, IHC can have problems with non-specific staining that can show false positive staining (Whitaker *et al.*, 2008).

RNAscope, the newest ISH method is a highly specific and sensitive method used to determine a single mRNA in tissue, using a standard microscope (Wang *et al.*, 2012). This method uses a novel double Z probe that binds to the target RNA and then to a cascade of a preamplifier, amplifier, and label probe (Wang *et al.*, 2012). This method provides high specificity and sensitivity detection due to using the double Z probe design which is able to control background staining (Wang *et al.*, 2012; Grabinski *et al.*, 2015). However, it is an expensive method. A previous study on a thick free-floating rodent brain sections and primary neuronal cultures suggested that a combination of RNAscope and IHC is a useful method that can be used to address a specific scientific question which is not readily answerable with a single technique (Grabinski *et al.*, 2015). Our data also suggest that the

use of both techniques on prostate tissues samples may give more accurate expression results than using only IHC staining.

6.3.3 The possible role of Sall4 in prostate cancer

Previous reports have proposed many different functional roles of Sall4 in cancer, including regulating cell transformation, proliferation, apoptosis, invasive migration and cell stemness (Zhang *et al.*, 2015). However, this section will focus on a couple of mechanisms for Sall4 which could explain its a role in PCa.

It has been found that cell proliferation and colony formation in the si-Sall4 group was significantly lower than in normal controls. In contrast, the apoptotic rate of the si-Sall4 group was higher than the control group. This study is also found increases in the pro-apoptotic protein Bax and decreased anti-apoptotic protein Bcl2 levels in the si-Sall4 group compared to normal control, using PCa cell lines (Liu and Shan, 2016). These results suggest that increased Sall4 in PCa might play a role in increasing the tumour formation by inhibiting apoptosis through Bcl2 and Bax proteins.

Sall4 might have another possible role in PCa. Sall4 could play a transcriptional activator role for the Bmi-1 gene which plays a role in protecting cells from apoptosis as well as in regulating differentiation and self- renewal of SCs (Jiang *et al.*, 2009; Wang *et al.*, 2014b). It has been shown that Sall4 can bind to the promoter region of Bmi-1 and regulate its expression. In addition, the Bmi-1 level in Sall4 transgenic mice was higher than wild-type controls and showed also a significant association between Sall4 reduction by siRNA in leukemic cell line and Bmi-1 level, suggesting there is a strong association between them in haematopoietic and leukemic cells (Yang *et al.*, 2007). These results, when taken together, indicate that Sall4 could play a role in PCa initiation through its role in activating Bmi-1 which promotes proliferation and inhibits apoptosis of cells. Undoubtedly, further work will shed more light on the possible role of Bmi-1 in PCa intention and progression as well as its association with Sall4.

PTEN is a tumour suppressor gene and essential in PCa (Jamaspishvili *et al.*, 2018). PTEN converts phosphatidylinositol-3,4,5-trisphosphate (PIP3) to phosphatidylinositol-4,5-trisphosphate (PIP2), thereby directly inactivating the PI3K/AKT pathway (Leevers *et al.*, 1999), whereas, losing its function which has been reported in cancers such as PCa, leads to activation of the PI3K/AKT pathway which then regulates various of cellular processes, including increased cell survival, proliferation and inhibiting of apoptosis (Li and Sun, 1998;

Planchon *et al.*, 2008). In addition to its role in promoting proliferation and apoptosis, nuclear PTEN shows an important role in chromosome stability, DNA repair, cell cycle arrest and cellular stability (Planchon *et al.*, 2008). Interestingly, decreased PTEN expression is found in Sall4 B transgenic mice compared to wild-type control, suggesting that Sall4 inhibits the transcription of PTEN (Lu *et al.*, 2009), suggesting increased Sall4 might play an important role in PCa cell formation through its a PTEN inhibitory role.

In human acute myeloid leukaemia, Sall4 is proposed to have a co-activator role of Wnt/β-catenin pathway through its direct interaction with β-catenin and enhancing synergistically the transcriptional output of the Wnt/β-catenin pathway (Ma *et al.*, 2006). Other studies reported that Sall4 could bind directly to c-Myc or TCF/LEF in Wnt/ β-catenin and then activate its expression (Bohm *et al.*, 2006; Li *et al.*, 2015a), suggesting Sall4 could contribute to PCa formation through activation of the Wnt/β-catenin pathway.

It is also important to consider that Sall4 appears to be increased in PCa compared to NP but decreased in higher grade tumours. This suggests that Sall4 could play a different role in tumour initiation than in tumour progression. Sall4 might play a role in regulating cell differentiation in PCa. A previous Sall4 immunohistochemical study on normal and malignant colon tissues proposed that increased Sall4 might promote cell differentiation in colorectal carcinoma (Hao *et al.*, 2016). This result is consistent with our Sall4 data which showed decreased Sall4 protein and mRNA level with increasing primary Gleason grade, using two different antibodies and mRNA probe. Taken together, increased Sall4 might play a role in PCa differentiation.

6.3.4 Conclusion

In conclusion, Sall4 expression appears to be increased in PCa compared to NP tissues and its expression is negatively associated with increasing primary Gleason grade, but is not associated with clinical stage and risk of biochemical relapse. Sall4 might have a role in promoting cell proliferation and differentiation and loss of its expression could be a potential indicator for aggressive PCa. Further studies are needed to confirm if there is any association of Sall4 with biochemical recurrence. Additional future work is considered in the final discussion.

CHAPTER SEVEN

IDENTIFICATION AND ASSESSMENT OF ZSCAN4 EXPRESSION IN PROSTATE TISSUES FROM THE BATH AND TMA COHORTS

7. Identification and Assessment of Zscan4 expression in prostate tissues from the Bath and TMA cohorts

7.1 Introduction

Zinc finger and SCAN domain containing 4 (Zscan4) is one of the candidate biomarkers that were identified for analysis. This chapter focuses on Zscan4 and describes why the protein was selected for analysis and presents all the experimental data that was collected for the protein.

7.1.1 Identification of Zscan4 using the literature searching

Zscan4 is thought to be a potential transcription factor (Falco *et al.*, 2007) and is expressed in ESCs and plays an important role in regulating telomere length and genomic stability in ESCs (Zalzman *et al.*, 2010; Ko *et al.*, 2013). The previous study found that Zscan4 is expressed predominantly in mouse ESCs and late two-cell embryo stage, suggesting that it might play an important role in preimplantation development (Falco *et al.*, 2007).

There is also some evidence for expression of Zscan4 in adult tissues. An IHC study showed nuclear and cytoplasmic Zscan4 staining in a small number of human pancreatic tissues, including islets of Langerhans, acini, ducts and oval-shaped cells, and its expression was increased in chronic pancreatitis (Ko *et al*., 2013b). Second, a non-published study by a PhD student in our laboratory (Ben Sharpe) showed nuclear Zscan4 staining was increased in a subset of PCa tissues compared to those with a normal nature (Sharpe, 2016). Nuclear Zscan4 was also found to be higher in biochemical recurrence tissues compared to non-recurrence (Sharpe, 2016), although this result was non-significant, perhaps because of the small sample size. Therefore, previous work in our laboratory suggested that Zscan4 expression might be linked to PCa formation and possibly biochemical relapse.

Given the evidence, described above, to suggest a link between Zscan4 staining and PCa and a potential role for the protein in SCs, it was decided to examine the expression of the protein in more detail. The hypothesis used was based on the recent study from our lab (Sharpe, 2016) and predicted that nuclear Zscan4 staining would be increased in PCa and might be positively associated with PCa relapse. The key publications and hypothesis for Zscan4 are summarised in Table 7.1

Table 7. 1 The key publications and hypothesis of Zscan4.

Name & type of Protein	Key publications	Hypothesis
ZSCAN4 **A potential transcription factor.**	❖ Nuclear and cytoplasmic Zscan4 staining was increased in chronic pancreatitis compared to normal pancreatic tissues (Ko *et al.*, 2013). ❖ Nuclear Zscan4 was overexpressed in a subset of PCa tissues compared to NP tissues and was not associated significantly with PCa histopathological parameters, including Gleason grades and relapse (Sharpe, 2016). However, nuclear Zscan4 H-scores were found to be higher in patients with PCa relapsed compared to those with non relapsed, suggesting that small sample size may explain this non-significant result.	1. Nuclear Zscan4 staining will be increased in a subset of PCa compared to NP tissues. 2. Nuclear Zscan4 staining could be positively associated with relapse.

7.1.2 Aims

The goal of this chapter is to test the hypothesis, described above by carrying out the following:

1. To determine the expression of Zscan4 in normal and malignant prostate tissues from the Bath and TMA cohorts.
2. To establish if the expression of Zscan4 correlates with histopathological and clinical features of PCa, including primary Gleason grade, clinical stage and biochemical recurrence.
3. To confirm the specificity of the Zscan4 IHC staining using RNAscope® to measure the expression of Zscan4 mRNA.

7.2 Results

7.2.1 Testing of antigen retrieval methods

Having chosen Zscan4 as a candidate biomarker, the next step was to assess its expression using IHC. Before this could be carried out, different types of antigen retrieval were tested to establish which gave the best staining for Zscan4.

IHC staining was carried out with an anti-Zscan4 antibody from Novus Biological that is referred to here as the anti-Zscan4 A antibody. IHC staining showed nuclear Zscan4 staining in prostate tissue using citrate (pH 6) and Tris / EDTA (pH 9) buffers. A citrate buffer was chosen for Zscan4 because it showed clear nuclear staining with less little significant background staining than a Tris/EDTA buffer (Figure 7.1).

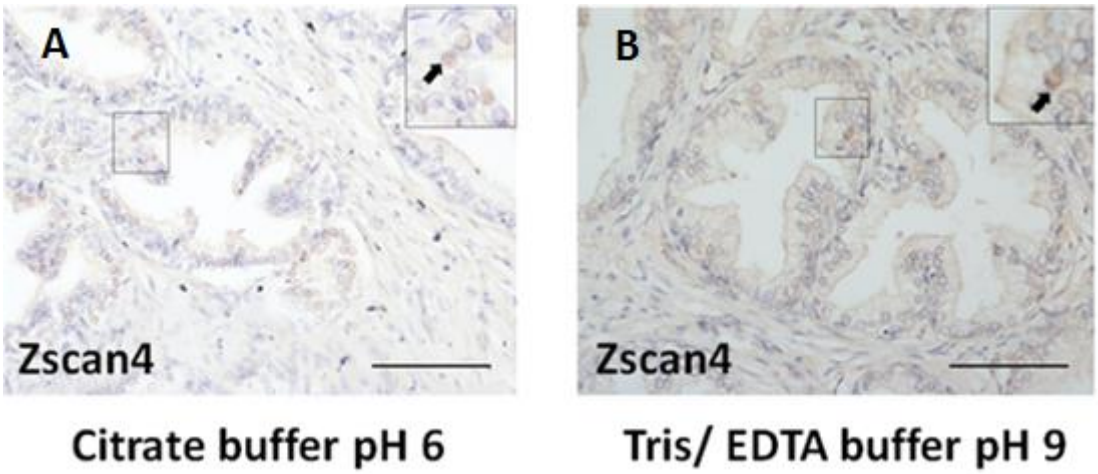

Figure 7. 1 Expression of Zscan4 after the different antigen retrieval methods. (A) Expression of Zscan4 in citrate buffer pH 6. (B) Expression of Zscan4 in Tris/EDTA buffer pH 9. IHC staining with citrate buffer showed a clearer nuclear Zscan4 staining compared to Tris/EDTA buffer. Scale bar=100μm with inserts at 2x zoom.

7.2.2 Expression of Zscan4 in the Bath cohort, using the anti-Zscan4 A antibody

To test the hypothesis that nuclear Zscan4 will be increased in a subset of PCa and will be positively associated with biochemical recurrence; IHC was carried out with the Bath cohort prostate tissues using the anti- Zscan4 A.

7.2.2.1 Immunohistochemical expression of anti-Zscan4 A in the Bath prostate samples

IHC was carried out to investigate the expression of Zscan4 on normal and malignant prostate tissue samples from the Bath cohort, using the anti-Zscan4 A antibody. IHC showed that NP tissues had, at low frequencies, nuclear anti-Zscan4 staining with different levels of staining patterns, ranging from moderate (Figure 7.2 A, arrow) to negative (Figure 7.2 B, arrow). Nuclear anti-Zscan4 staining was also observed in the glandular epithelium of PCa and the intensity of signal varied widely, from strong and widespread (Figure 7.2 C, arrow), moderate and scattered (Figure 7.2 D, arrow), weak at low frequency (Figure 7.2 E, arrow) and negative (Figure 7.2. F, arrow).

In addition to the nuclear staining, cytoplasmic Zscan4 staining was found in normal and malignant prostate tissues with different levels of staining ranging from moderate (Figure 7.2 C, arrowhead) to weak (Figure 7.2 A, arrowhead) or negative (Figure 7.2 F). Zscan4 is expressed in placenta tissues (Sharpe, 2016) and so this study used normal placental tissue as a positive control for anti-Zscan4 A and as expected IHC showed nuclear anti-Zscan4 A

staining in normal placental tissue (Figure 7.2 G, arrows). A negative control showed no detectable background staining in prostate tissue (Figure 7.2 H, arrow).

7.2.2.2 Association between anti-Zscan4 A immunostaining and histopathological and clinical parameters of prostate cancer in the Bath cohort

The previous PhD student, Benjamin Sharpe, stained 27 PCa vs. 5 NP tissues from the Bath cohort. This study added 7 PCa new samples to his data to increase the sample size. Data from both studies are included in the results presented below.

Having carried out IHC staining, the nuclear anti-Zscan4 A staining was quantified using the H-score system (Described in Chapter 2.2.2) and then compared to PCa histopathological and clinical parameters. The range of nuclear anti-Zscan4 A H-score in the Bath cohort varied between 0- 60 (Figure 7.3). The potential association between anti-Zscan4 A IHC results and histopathological and clinical parameters of PCa was then analysed and is described below.

The first analysis looked at the expression of anti-Zscan4 A in normal vs. malignant prostate tissues. Quantification of the IHC staining showed nuclear anti-Zscan4 staining expressed in 87% of PCa tissues compared to a very little expression level in NP tissues, but the result was not significant (P= 0.2899) (Figure 7.3 A & Table 7.2). There was no significant association between nuclear Zscan4 staining and PCa histopathological and clinical parameters (Figure 7.3 B, C&D &Table 7.2).

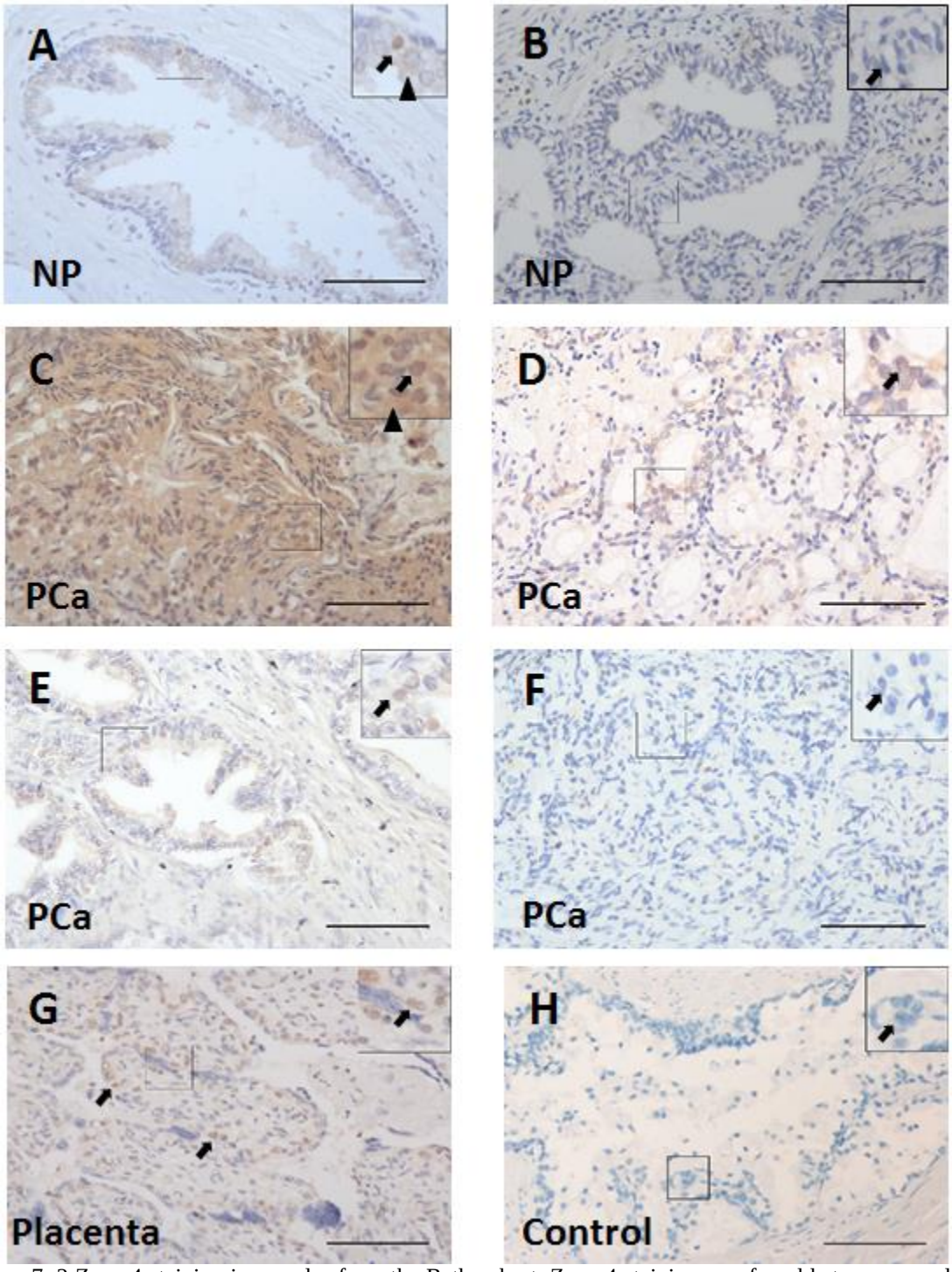

Figure 7. 2 Zscan4 staining in samples from the Bath cohort. Zscan4 staining was found heterogeneously in both normal and malignant tissues of the prostate, using anti-Zscan4 A antibody. (A) Moderate nuclear (Black arrow) and weak cytoplasmic (Black arrowhead) anti-Zscan4 A staining in NP. (B) NP had no anti-Zscan4 A staining. (C) Moderate nuclear (Black arrow) and cytoplasmic (Black arrowhead) anti-Zscan4 A staining in PCa. (D) Moderate nuclear (Black arrow) anti-Zscan4 A staining in PCa. (E) Weak nuclear (Black arrow) anti-Zscan4 A staining in PCa. (F) Negative anti-Zscan4 A staining in PCa. (H) Positive control: Nuclear anti-Zscan4 A staining (Black arrows) in placenta tissue. (H) Negative control (no primary antibody added) was free from background staining (Black arrow) in PCa. Scale bars=100µm with inserts at 2x zoom.

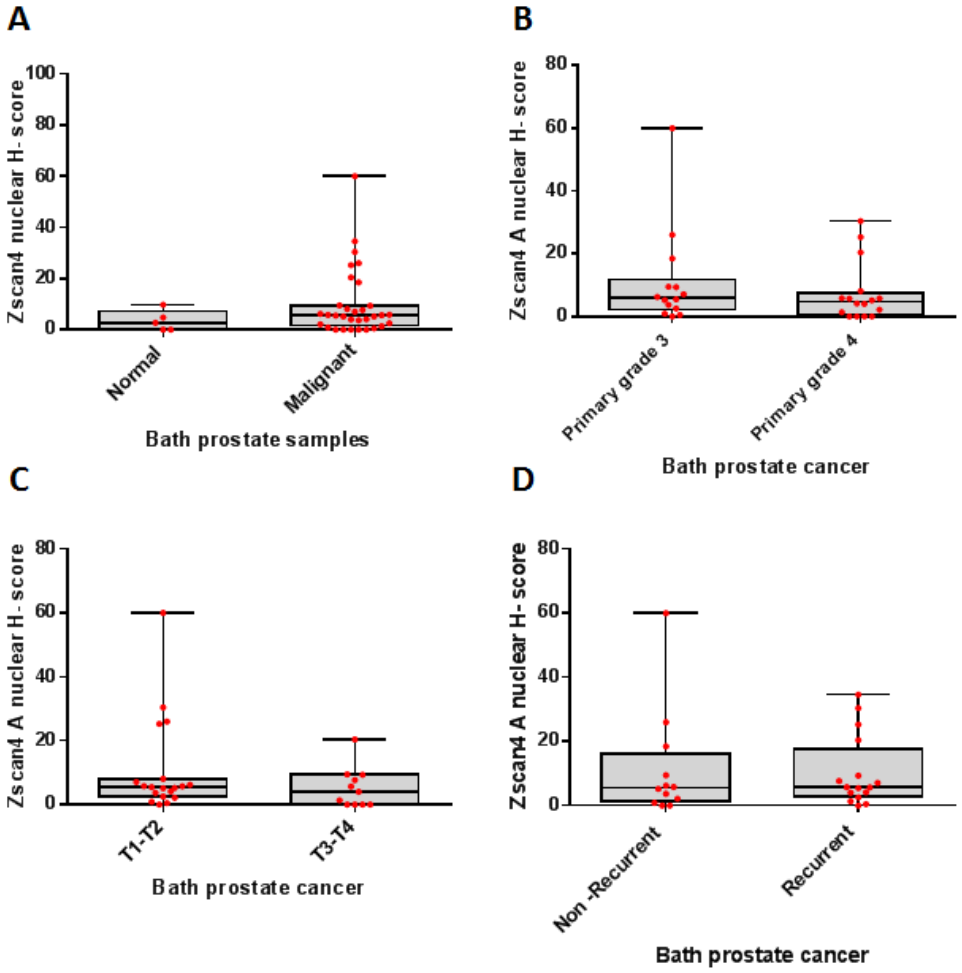

Figure 7. 3 Quantification of nuclear Zscan4 staining in both normal and malignant Bath prostate tissues, using anti-Zscan4 A antibody. Immunohistochemical staining of anti-Zscan4 A was quantified in the Bath cohort using H-score for nuclear IHC staining. (A) Nuclear anti-Zscan4 A staining was increased in PCa compared to NP (B) Nuclear anti-Zscan4 A staining was not associated with primary Gleason grade (p= 0.2899) (C) Nuclear anti-Zscan4 A staining was not associated with primary tumour stage T (p=0.2567). (D) Nuclear anti-Zscan4 A staining was not associated with biochemical relapse (p= 0.8159). The mean of five random fields was taken per prostate sample. Statistical significance was determined with unpaired T-test for each set of conditions. NP (n=4), PCa (n= 32), grade 3 (n=14), grade 4 (n=16), T1-2 (n=19), T3-4 (n= 11), recurrent (n=16) and non-recurrent (n=12).

Table 7. 2 Nuclear anti-Zscan4 A staining results with Bath cohort clinical data.

Comparison	**Nuclear anti-Zscan4 A staining**	
	Results	**p. value**
Normal vs malignant	No statistically significant difference	0.2899
Gleason grades (Grade 3 & 4)	No statistically significant difference	0.4388
Stage (T)	No statistically significant difference	0.2567
5 years Biochemical recurrence	No statistically significant difference	0. 8159

In summary, there was no significant difference between nuclear anti-Zscan4 A staining in normal vs. malignant prostate tissues. Nuclear anti-Zscan4 A staining showed also no associated with primary Gleason grade, clinical stage and biochemical recurrence. This result should be treated as preliminary data because of the sample size, so the staining was repeated using a tissue microarray with a larger sample size.

7.2.3 Expression of Zscan4 in the TMA cohort, using the anti-Zscan4 A antibody

7.2.3.1 IHC staining of anti-Zscan4 A in the TMA prostate samples

IHC was carried out on prostate tissues from the TMA cohort. IHC staining showed that Zscan4 staining was not detected in NP tissues, using anti-Zscan4 A (Figure 7.4 A, arrow). In contrast, nuclear anti-Zscan4 A staining was found in a subset of PCa tissues and the intensity of signal varied widely, from strong and widespread (Figure 7.4 B, arrow), moderate (Figure 7.4 C, arrow), weak (Figure 7.4 D, arrow) and negative (Figure 7.4 G, arrow). In addition, cytoplasmic anti-Zscan4 A staining was also observed in some cases of normal and malignant prostate tissue from the Bath cohort with different levels of staining. The different levels of cytoplasmic staining might be important, so quantification was included in further analysis. Cytoplasmic anti-Zscan4 A staining was also observed in some PCa tissues with different levels of staining patterns, ranging from strong (Figure 7.4 E, arrowhead) to weak (Figure 7.4 F, arrowhead) or negative (Figure 7.4 G, arrow). A negative control showed no clear background staining in PCa tissue (Figure 7.4 H, arrow).

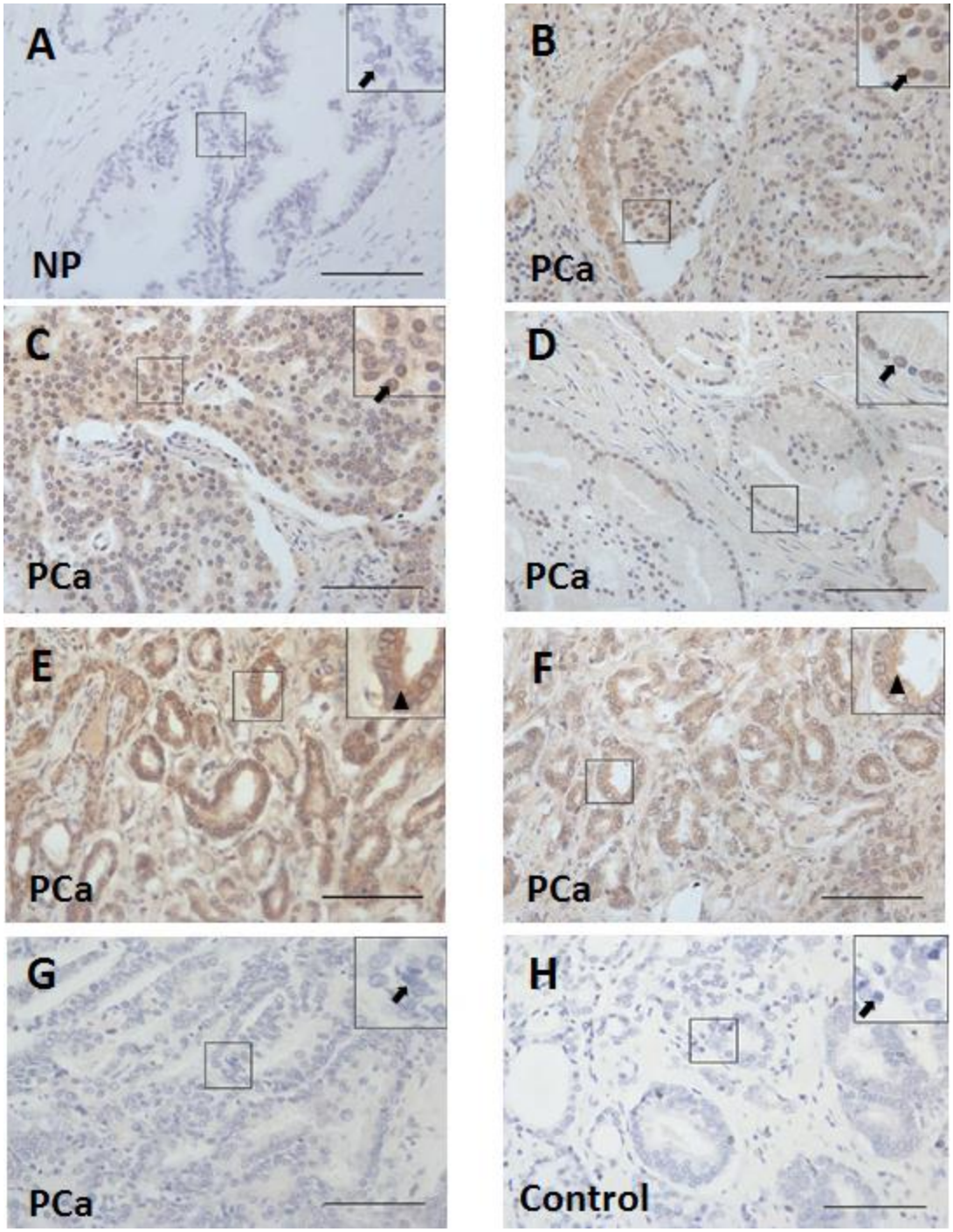

Figure 7. 4 Zscan4 expressions in samples from the TMA cohort. Zscan4 A was expressed heterogeneously in both normal and malignant tissues of the prostate, using anti-Zscan4 A antibody. (A) Negative anti-Zscan4 A staining in NP. (B) Strong nuclear (Black arrow) anti-Zscan4 A staining in PCa. (C) Moderate nuclear (Black arrow) anti-Zscan4 A staining in PCa. (D) Weak nuclear (Black arrow) anti-Zscan4 A staining in PCa. (E) Strong cytoplasmic anti-Zscan4 A staining (Black arrowhead) in PCa. (F) Weak cytoplasmic anti-Zscan4 A staining (Black arrowhead) in PCa. (G) PCa tissue had no clear anti-Zscan4 A staining. (H) Negative control (no primary antibody added) shows no clear background staining (Black arrow) in PCa. Scale bars=100µm with inserts at 2x zoom.

7.2.3.2 Association between anti-Zscan4 A immunostaining with histopathological parameters of prostate cancer in the TMA cohort

In order to evaluate, if there was an association between anti-Zscan4 A staining in normal and malignant prostate tissues, different primary Gleason grades, clinical stages and biochemical recurrence, nuclear and cytoplasmic anti-Zscan4 A staining was assessed using the H & proportion and intensity 1 scores, respectively and then compared to histopathological variables accompanying the TMA. The range of nuclear and cytoplasmic anti-Zscan4 A scores in the TMA varied between 0-85.5 and 0-6, respectively (Figure 7.5).

Quantification of the IHC staining showed nuclear anti-Zscan4 A staining expressed in a subset of PCa tissues (21.3%), whereas, all NP tissues had negative staining for anti-Zscan4 A. However, this result showed no significant difference between groups (p= 0.1076) (Figure 7.5 A & Table 7.3). Cytoplasmic anti-Zscan4 staining was increased significantly in a subset of PCa compared to NP tissues (p= 0.01) (Figure 7.5 B & Table 7.3).

There was no significant difference in the mean of nuclear anti-Zscan4 A staining among primary Gleason grade groups (p= 0.4221) (Figure 7.4 C & Table 7.2). In contrast, cytoplasmic anti-Zscan4 A staining showed a significant difference between primary Gleason grade groups (P = 0.0487) (Figure 7.4 D & Table 7.2). Analysis of the IHC data, using multiple comparisons Tukey's tests showed cytoplasmic anti-Zscan4 A staining was reduced significantly when comparing PCa patients with a Gleason grade 5 to those with a Gleason grade 4 (p=0.046), but no significant difference between Gleason grade 3 tissues to grade 4 (P=0.1644) or grade 5 tissues (p=0.9994). Nuclear and cytoplasmic anti-Zscan4 A staining was not associated significantly with clinical stage (TNM) (Table 7.3).

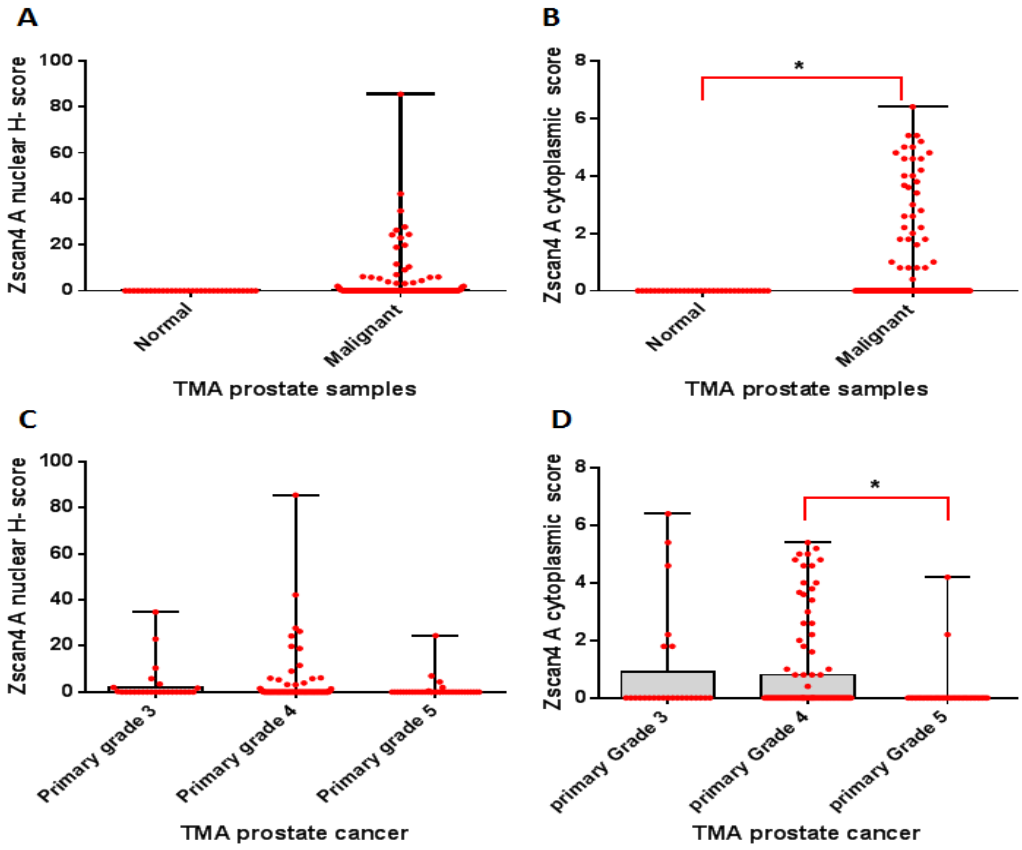

Figure 7. 5 Quantification of nuclear and cytoplasmic anti-Zscan4 A staining in both normal and malignant TMA prostate tissues. Immunohistochemical staining of anti-Zscan4 A was quantified in the TMA cohort using H-score and proportion and intensity 1 for nuclear and cytoplasmic IHC staining respectively. (A) Nuclear anti-Zscan4 A staining was expressed in a subset of PCa (p=0.1076). (B) Cytoplasmic anti-Zscan4 A was increased significantly in PCa compared to NP (p= 0.01). (C) Nuclear anti-Zscan4 A was not associated significantly with primary Gleason grade (p=0. 4221). (D) Cytoplasmic anti-Zscan4 A staining showed a significant difference among Gleason grade groups (p=0.0487). Multi comparison tests showed a significant reduction in the cytoplasmic anti-Zscan4 scores when comparing Gleason grades 5 tissues to Gleason grade 4 (p=0.046). Data represent the mean of five random images per case. Unpaired t-test or one-way ANOVA with multi-comparison (Tukey) tests was conducted for two or three groups respectively to determine the statistical difference for each set of conditions. NP (n=16), PCa (80), grade 3 (n=13), grade 4 (n=46) and grade 5 (n=18).

Table 7. 3 Nuclear anti-Zscan4 A staining results with the TMA clinical data.

Comparison	**Nuclear anti-Zscan4 A staining**			**Cytoplasmic anti-Zscan4 A staining**		
	Results		**p. value**	**Results**		**p. value**
Normal vs malignant	increased in a subset of PCa, but no significant difference		0.1076	increased in a subset of PCa		0.01
Gleason grades (3, 4 &5)	No statistically significant difference	ANOVA test	0.4221	Lower in high grades	ANOVA test	0.487
		Grade 4 vs. Grade 3	0.9974		Grade 4 vs. Grade 3	0.9994

		Grade 5 vs. Grade 3	0.6341		Grade 5 vs. Grade 3	0.1644
		Grade 5 vs. Grade 4	0.4031		Grade 5 vs. Grade 4	0.046
Stage (T)	No statistically significant difference		0.1146	No statistically significant difference		0.2764
Stage (M)	No statistically significant difference		0.1861	No statistically significant difference		0.1534
Stage (N)	No statistically significant difference		0.1676	No statistically significant difference		0.5412

In summary, nuclear and cytoplasmic anti-Zscan4 staining was increased in a subset of PCa compared to NP tissues, although the nuclear staining results were not significant. Nuclear and cytoplasmic anti Zscan4 A staining was not associated significantly with primary Gleason grade and clinical stage (except for cytoplasmic staining between grade 4 & 5).

7.2.4 Expression of Zscan4 in the Bath cohort using the anti-Zscan4 B antibody

In order to show that the anti-Zscan4 A staining reported above was accurately showing changes in Zscan4 protein expression, the results should be repeated with an independent antibody. To achieve this a second independent Zscan4 rabbit polyclonal antibody from Abcam, that is referred to here as the anti-Zscan4 B, was identified.

The original antibody, anti-Zscan4 A is a mouse polyclonal antibody, but the provider did not provide us with information about its immunogen (Figure 7.7 A). The second antibody, anti-Zscan4 B is a rabbit polyclonal raised with an immunogen that was a fragment of the C-terminal of Zscan4 (Figure 7.7 B). Therefore, although we do not know if the two antibodies come from different regions of the protein, the two antibodies were raised independently (shown by the fact they have different hosts) and so the second antibody was chosen for testing on normal placenta and PCa tissues from the Bath cohort, to show if it gave similar results to the first antibody.

Human Zscan4 protein

```
  1 maldlrtifq cepsennlgs ensafqqsqg pavqreegis efsrmvlnsf qdsnnsyarq
 61 elqrlyrifh swlqpekhsk deiisllvle qfmigghcnd kasvkekwks sgknlerfie
121 dltddsinpp alvhvhmqgq ealfsedmpl rdvivhltkq vnaqttrean mgtpsqtsqd
181 tsletgqgye deqdgwnsss kttrvnenit nqgnqivsli iiqeengprp eeggvssdnp
```

241 ynskraelvt arsqegsing itfqgvpmvm gagcisqpeq sspesalthq snegnstcev
301 hqkgshgvqk sykceecpkv fkylchllah qrrhrnerpf vcpecqkgff qisdlrvhqi
361 ihtgkkpftc smckksfshk tnlrsherih tgekpytcpf cktsyrqsst yhrhmrthek
421 itlpsvpstp eas

A. Zscan4 mouse polyclonal antibody

The provider does not specify which part of human Zscan4 the immunogen originated from

B. Zscan4 rabbit polyclonal antibody

361 ihtgkkpftc smckksfshk tnlrsherih tgekpytcpf cktsyrqsst yhrhmrthek
421 itlpsvpstp eas

Figure 7. 6 Zscan4 proteins sequence with the immunogenic parts of the two different antibodies highlighted. (A) Human Zscan4 protein consists of 433 amino acid and the antibodies were raised independently as they have different hosts or clonity. (A) Zscan4 mouse polyclonal (anti-Zscan4 A) immunogen is not provided by the company. (B) Zscan4 rabbit polyclonal (anti-Zscan4 B) immunogen was a partial fragment of the C-terminal of Zscan4 (Grey highlighted).

7.2.4.1 Immunohistochemical staining patterns of Zscan4 antibodies on the placenta and prostate tissues from the Bath cohort

IHC was carried out using the two different Zscan4 antibodies (anti-Zscan4 A& B) on sections from similar regions of the placenta and prostate tissue samples from the Bath cohort. Strong to weak scattered nuclear (Figure 7.7 A, arrows) and weak cytoplasmic (Figure 7.7 A, arrowhead) anti-Zscan4 A staining was observed in placental tissue, whereas, strong and widespread nuclear (Figure 7.7, B, arrows) and weak cytoplasmic (Figure 7.7 B, arrowhead) anti-Zscan4 B staining was shown in placental tissue.

Zscan4 was expressed weakly in the cytoplasm of the glandular regions of PCa, using anti Zscan4 A antibody (Figure 7.7 C, arrowhead). In contrast, strong nuclear (Figure 7.8 D, arrows) and cytoplasmic (Figure 7.7 D, arrowhead) anti-Zscan4 B staining was detected in PCa tissue. Another PCa section showed no clear anti-Zscan4 A staining (Figure 7.7 E, arrows), whereas, the similar section from same PCa case had strong nuclear anti-Zscan4 B staining (figure 7.7, F, arrow).

The two different Zscan4 antibodies (A&B) had different staining patterns in both placental and prostate tissue samples. So it was decided not to use anti-Zscan4 B for further analysis. Rather than test a third antibody, it was decided to use RNAscope to visualise Zscan4 mRNA expression patterns and thus provide more insight into which staining pattern was likely to be correct.

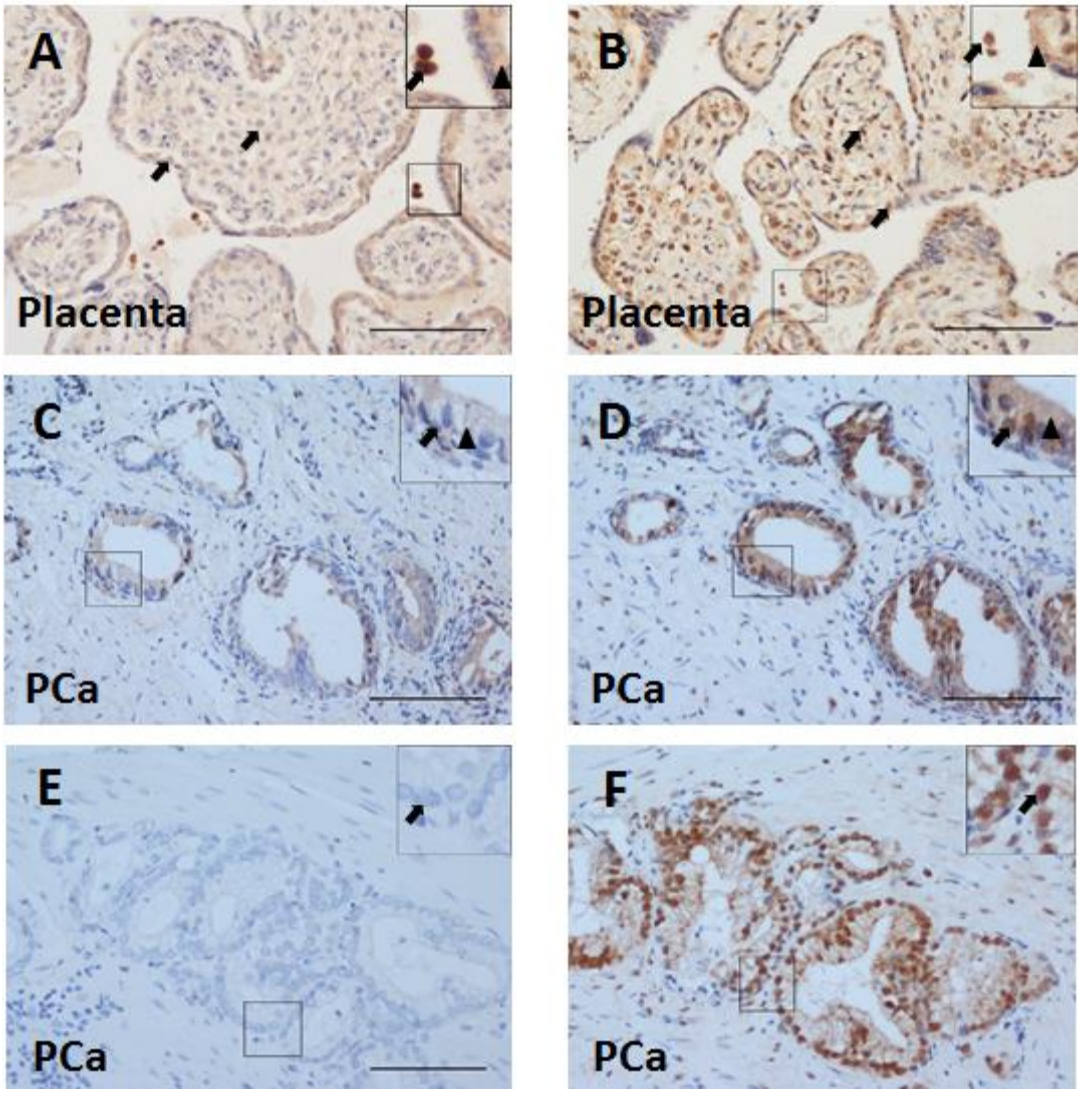

Figure 7. 7 Immunohistochemical staining of using two distinct Zscan4 antibodies in the Bath placental and prostate tissue samples. (A) Strong to weak nuclear (Black arrows) and weak cytoplasmic (Black arrowhead) anti-Zscan4 A in placental tissues. (B) Strong nuclear (Black arrows) and cytoplasmic (Black arrowhead) anti-Zscan4 B in placental tissue. (C) Weak nuclear (Black arrow) and cytoplasmic (Black arrowhead) anti-Zscan4 A in PCa. (D) Strong nuclear (Black arrow) and cytoplasmic (Black arrowhead) anti-Zscan4 B in PCa. (F) Negative staining for anti-Zscan4 A in PCa. (F) Strong nuclear (Black arrow) anti-Zscan4 B in PCa. Both antibodies showed different staining patterns in placenta and prostate tissues. Scale bars—100 μm with inserts at 2x zoom.

7.2.5 Analysis of expression of Zscan4 mRNA using RNAscope®

7.2.5.1 Zscan4 mRNA expression in prostate tissues from the Bath cohort

RNAscope® was carried out on prostate tissue samples from the Bath cohort. The result showed no significant staining for Zscan4 mRNA in all NP tissues (Figure 7.9 A, arrow). In contrast, Zscan4 mRNA staining was observed in PCa tissues and the number of positive cells varied, from few positive dots per cell (Figure 7.8 B&C, arrows) to negative (Figure 7.8 D, arrow). A positive control tissue, placenta showed Zscan4 mRNA staining (Figure 7.8, E, arrows). The negative control probe showed no background staining in PCa tissue (Figure 7.8 H, arrow).

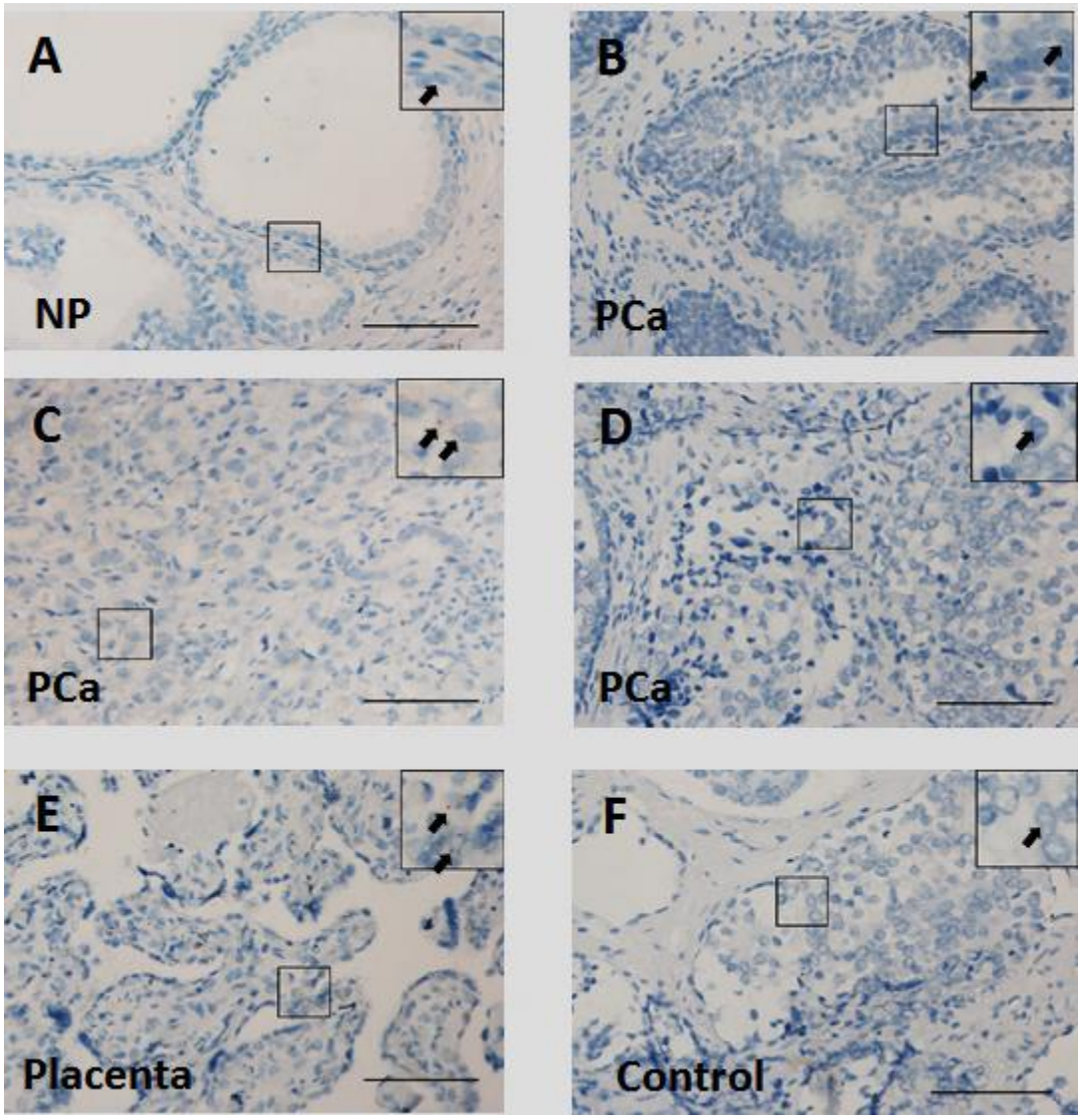

Figure 7. 8 Zscan4 mRNA staining in prostate samples from the Bath cohort. (A) No Zscan4 mRNA staining in NP (Black arrow). (B) Zscan4 mRNA staining (Black arrows) in PCa. (C) Zscan4 mRNA staining (Black arrows) in PCa. (D) No Zscan4 mRNA staining (Black arrow) in PCa. (E) Zscan4 mRNA staining (Black arrows) in normal placental tissue. (F) Negative control showed a free background staining (Black arrow) in PCa. Scale bars=100µm with inserts at 2x zoom.

7.2.5.2 Association between Zscan4 mRNA staining and histopathological parameters of prostate cancer in the Bath cohort

Histologically normal and malignant prostate tissues were stained for Zscan4 mRNA and scored using the Zscan4 mRNA score (described in Chapter 2.4). The range of Zscan4 mRNA score in the Bath cohort varied between 0-0.4.

Statistical analysis was not performed because of the small sample size of the Bath cohort. However, the preliminary data showed that Zscan4 at the mRNA level was not expressed in all NP tissues, whereas, 33% of PCa had Zscan4 mRNA staining. There was no clear association between Zscan4 mRNA staining and other PCa histopathological parameters, including primary Gleason grade, clinical stage and biochemical relapse. This data is based on small sample size, so RNAscope® was repeated to measure the expression of Zscan4 at mRNA level in the large TMA cohort.

7.2.6 Zscan4 mRNA staining in prostate tissues from the TMA cohort

7.2.6.1 Zscan4 mRNA expression in prostate tissues from the TMA cohort

The RNAscope® result showed no significant Zscan4 mRNA staining in NP tissues (Figure 7.9 A, arrow), but it was expressed in a subset of PCa tissues and the number of positive cells varied widely, from a widespread (Figure 7.9 B&C, arrows), scattered (Figure 7.9 D, arrow) and negative (Figure 7.9 E, arrow). A negative control was free of background staining in prostate tissue (Figure 7.9 F, arrow). This result was consistent with the Zscan4 mRNA result in the Bath cohort.

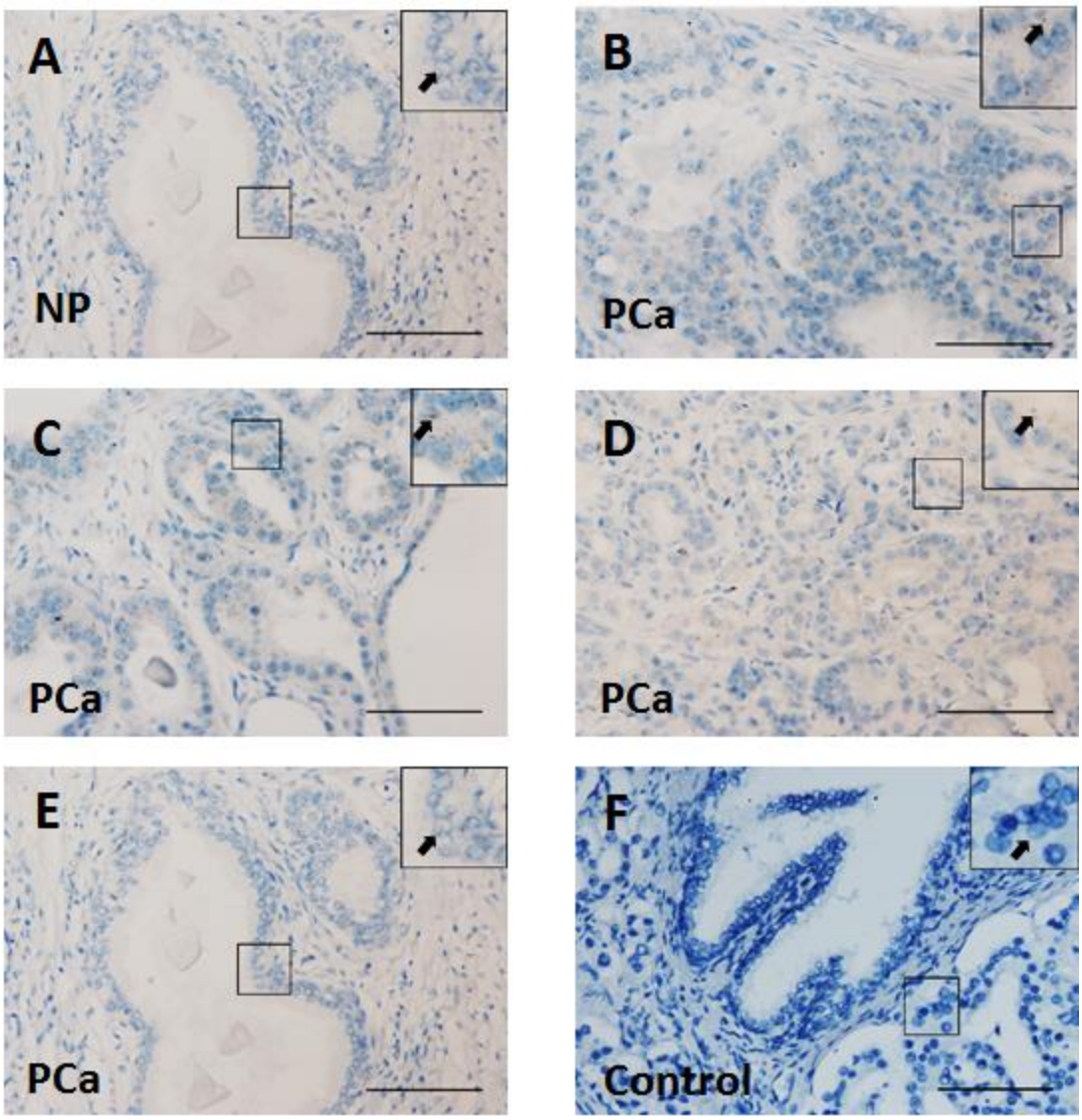

Figure 7. 9 Zscan4 mRNA staining in prostate samples from the TMA cohort. (A) NP tissue had negative staining for Zscan4 mRNA (Black arrow). (B) Zscan4 mRNA staining (Black arrow) in PCa. (C) Zscan4 mRNA staining (Black arrow) in PCa. (D) Zscan4 mRNA staining (Black arrow) in PCa. (E) No Zscan4 mRNA staining (Black arrow) in PCa. (F) Negative control showed a free background staining (Black arrow) in PCa. Scale bars=100µm with inserts at 2x zoom.

7.2.6.2 Association between Zscan4 mRNA staining and histopathological parameters of prostate cancer in the TMA cohort

Having carried out RNAscope® staining, Zscan4 mRNA staining was then quantified using the Zscan4 mRNA score (described in chapter 2.4). The range of Zscan4 mRNA score varied between 0-3 (Figure 7.10). The potential association between Zscan4 mRNA staining and histopathological parameters of PCa was then analysed and described below.

The first analysis looked at the mRNA expression of Zscan4 in normal vs. malignant prostate tissues. Quantification of the RNAscope® showed Zscan4 mRNA staining increased significantly in a subset of PCa compared to NP tissues (p=0.0465) (Figure 7.10 A& Table 7.5), but was not associated with other PCa histopathological parameters such as primary Gleason grade and clinical stage (Figure 7.10 B & Table 7.5).

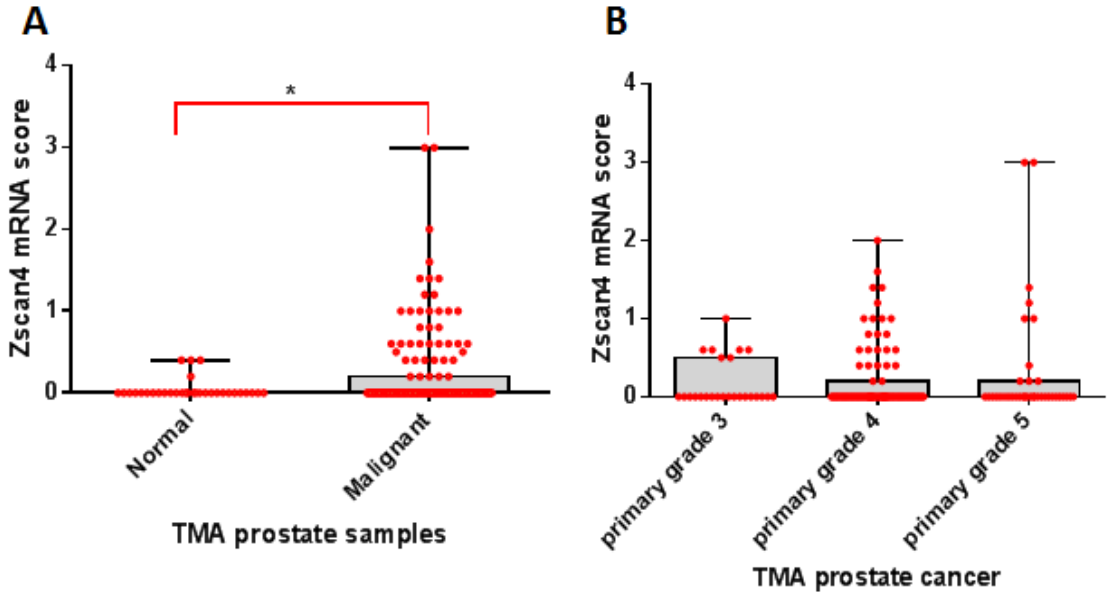

Figure 7. 10 Quantification of Zscan4 mRNA staining in both normal and malignant TMA prostate tissues. Zscan4 mRNA staining was quantified in the TMA cohort using Zscan4 mRNA score. (A) Zscan4 mRNA staining was increased significantly in a subset of PCa (p=0.0465). (B) There was no significant difference in the mean of Zscan4 mRNA score among Gleason grades (p=0.5136). Data represent the mean of five random images per case. Unpaired T-test or one-way ANOVA with Tukey's multi-comparison tests were conducted for two or three groups respectively to determine the statistical difference for each set of conditions. NP (n=16), PCa (80), grade 3 (n=13), grade 4 (n=46) and grade 5 (n=18).

Table 7. 4 Zscan4 mRNA staining results with the TMA clinical data.

Comparison	**Zscan4 mRNA staining**		
	Results		**p. value**
Normal vs malignant	Increased in a subset of PCa		0.0465
Primary Gleason grade (3, 4 &5)	No statistically significant difference	Anova test	0.5136
		Grade 4 vs. Grade 3	0.5781
		Grade 5 vs. Grade 3	0.5626
		Grade 5 vs. Grade 4	0.4070
Stage (T)	No statistically significant difference		0.4654
Stage (M)	No statistically significant difference		0.7817
Stage (N)	No statistically significant difference		0.927

In summary, Zscan4 mRNA staining was increased significantly in a subset of PCa compared to NP tissues, but was not associated with primary Gleason grade and clinical stage.

2.5.7 Zscan4 results in the Bath and TMA cohorts: testing the hypothesis

The results from the two cohorts and if the hypothesis is accepted or rejected is summarised in Table 7.5.

Table 7. 5 The summary of Zscan4 results in the Bath and TMA cohorts

Parameters	localisation	Hypothesis	Bath	TMA	Results Confirmed?	Hypothesis Accepted?
Normal vs Cancer	Nuclear Zscan4	Zscan4 will be increased in PCa	Increased in a subset PCa	Increased in a subset PCa	Yes	Yes
	Cytoplasmic Zscan4		Increased in a subset PCa	increased in a subset PCa	Yes	Yes
	Zscan4 mRNA		Increased in a subset PCa	Increased in a subset PCa	Yes	Yes
Primary Gleason grades	Nuclear Zscan4	Zscan4 will not be associated with grade	No significant difference	No significant difference	Yes	Yes
	Cytoplasmic Zscan4		No significant difference	No significant difference	Yes	Yes
	Zscan4 mRNA		No significant difference	No significant difference	Yes	Yes
Stage T	Nuclear Zscan4	Zscan4 will not be associated with stage	No significant difference	No significant difference	Yes	Yes
	Cytoplasmic Zscan4		No significant difference	No significant difference	Yes	Yes
	Zscan4 mRNA		No significant difference	No significant difference	Yes	Yes
Stage M	Nuclear Zscan4		Not tested	No significant difference	N/A	Yes
	Cytoplasmic Zscan4		Not tested	No significant difference	N/A	Yes
	Zscan4 mRNA		Not tested	No significant difference	N/A	Yes
Stage N	Nuclear Zscan4		Not tested	No significant difference	N/A	Yes

	Cytoplasmic Zscan4		Not tested	No significant difference	N/A	Yes
	Zscan4 mRNA		Not tested	No significant difference	N/A	Yes
Relapsed Vs. Non-relapsed	Nuclear Zscan4	Zscan4 will be associated with relapse	No significant difference	Not tested	N/A	NO
	Cytoplasmic Zscan4		No significant difference	Not tested	N/A	NO
	Zscan4 mRNA		No significant difference	Not tested	N/A	NO

7.3 Discussion

7.3.1 Summary

This study used IHC and RNAscope® to investigate the expression of Zscan4 protein and mRNA levels in normal and malignant prostate tissues from the Bath and TMA cohorts and to establish if there was an association between Zscan4 staining and PCa histopathological parameters, including primary Gleason grade, clinical stage and biochemical recurrence. Two different Zscan4 antibodies and an mRNA probe were used in this study. The staining patterns of Zscan4, using two different antibodies was different in prostate and placenta tissues form the Bath cohort. However, the mRNA staining and antibody results from the first antibody were consistent and confirmed that Zscan4 protein and mRNA levels were increased in a subset of PCa compared to NP, but were not associated significantly with primary Gleason grade, clinical stage and biochemical recurrence (Figure 7.6). In this chapter, I will discuss the key findings for Zscan4 and what is the possible role of Zscan4 in PCa.

Table 7. 6 Confirmed patterns of expression for Zscan4.

Zscan4	Confirmed patterns of expression
Anti-Zscan4 A	Nuclear and cytoplasmic anti-Zscan4 A staining was increased in a subset of PCa, but was not associated with primary Gleason grade, clinical stage and relapse.
Zscan4 mRNA	Zscan4 mRNA staining was increased in a subset of PCa, but was not associated with primary Gleason grade, clinical stage and biochemical relapse.

7.3.2 Zscan4 staining is increased in a subset of prostate cancer but is not associated with primary Gleason grade, clinical stage and biochemical recurrence.

Data presented in this chapter showed that Zscan4 staining at protein and mRNA levels were increased in a subset of PCa compared to NP. There was no significant association between Zscan4 staining and the histopathological parameters of PCa, including primary Gleason grade, clinical stage and biochemical recurrence.

There is no published study that has tested Zscan4 expression in cancer tissues, including PCa. However, Ko *et al*. showed nuclear and cytoplasmic Zscan4 staining in a small number of human pancreatic tissues, including islets of Langerhans, acini, ducts and oval-shaped cells, and its expression level was increased in chronic pancreatitis (Ko *et al.*, 2013). The previous unpublished work from our laboratory, carried out by Ben Sharp, showed nuclear Zscan4 increased in a subset of PCa compared to NP tissues. In contrast, nuclear Zscan4 staining was not associated significantly with other PCa histopathological and clinical features such as Gleason score and relapse. The data presented here is consistent with the previous data from Ben Sharpe (Sharpe, 2016), suggesting Zscan4 might have a role in PCa formation. Although my data showed no significant association between Zscan4 protein and mRNA levels with a risk of relapse, there are still many questions about this association because of the limited numbers of samples in the Bath cohort and no data concerning biochemical recurrence in the TMA cohort. It will be very interesting to assess association in a large cohort with clinical information about PCa recurrence, using IHC and RNAscope®. Additional future work is considered in the final discussion.

7.3.3 The possible role of Zscan4 in prostate cancer

In 2007, Zscan4 was shown to be expressed in late 2 cell embryos and embryonic SCs, but its functional mechanism remained unknown (Falco *et al*., 2007). However, a study in 2010 done by Zalzman, *et al* reported that Zscan4 has a role in regulating telomere elongation and genomic stability and its knockdown showed telomere shortening, increased karyotype abnormalities and sister chromatid exchange (Zalzman *et al*., 2010). Two further studies on different cell lines also reported that Zscan4 may have a role in maintaining genomic stability by regulating telomere elongation, but this regulation was in independent of the telomerase maintenance pathway (Lee and Gollahon, 2014, 2015). There is a direct interaction between Zscan4 and a repressor-activator protein 1 (Rap1), one of the shelterin complex (telomere

binding factors) and this direct binding may play a role in maintaining telomere length (Lee and Gollahon, 2014).

This evidence for a role for Zscan4 in maintaining telomere length and genomic stability raises the possibility that increased Zscan4 in cancer cells might also lead to telomere defects and potentially genomic instability. In addition, a previous study demonstrated that Zscan4 was positively regulated by p110a isoform of PI3-Kinase (Storm *et al*., 2014). The PI3K-AKT pathway is activated by the loss of PTEN which is found to be strongly associated with adverse oncological outcomes of PCa (Jamaspishvili *et al.*, 2018), suggesting that Zscan4 could be activated by the PI3K pathway in PCa.

7.3.4 Conclusion

In conclusion, Zscan4 appears to be overexpressed in a subset of PCa compared to NP tissues, but its expression was not associated significantly with primary Gleason grade, clinical stage and biochemical relapse. Further study needs to test if there is an association between Zscan4 expression and risk of relapse in samples from a larger cohort of patients and elucidate its molecular function in normal and malignant prostate tissues. Additional future work is considered in the final discussion.

CHAPTER EIGHT

FINAL DISCUSSION & FUTURE RESEARCH

8. Final discussion and future research

8.1 Introduction

The major goal of this study was to identify proteins that are differentially expressed between normal and malignant prostate tissues and/or between different Gleason grades, clinical stages and recurrence vs non-recurrence PCa. This is important to improve our understanding of the molecular basis of PCa formation and progression and potentially help in the development of future biomarkers. To achieve this goal, the project involved the identification and evaluation of the expression of proteins in prostate samples from two independent cohorts of patients using IHC and RNAscope®.

8.2 Main conclusions

Eight potential biomarkers were identified using literature searching and their expression evaluated by IHC and /or RNAscope®. β-catenin, Sox7, ABCG2, and membranous and cytoplasmic NDRG1 staining was decreased in PCa compared to NP tissues, whereas, Sall4, Zscan4, nuclear NDRG1 and glandular ALDH1A1 staining was increased in PCa. There was a negative association between the expression of markers β-catenin, Sox7, ABCG2 and Sall4, and primary Gleason grade and a positive association was observed between nuclear NDRG1 and primary Gleason grade. Finally, three of these biomarkers (Sox7, ABCG2 and stromal ALDH1A1) were negatively associated with biochemical relapse. In this chapter, I will focus on the four findings I believe to be of most significance.

- The expression of Sox7, ABCG2 and stromal ALDH1A were negatively associated with biochemical relapse.
- Sox7, β-catenin and ABCG2 expression was reduced in PCa compared to NP and was negatively associated with increasing Gleason grade.
- Sall4 at protein and mRNA levels were increased in PCa compared to NP tissues, but the expression of Sall4 was associated negatively with increasing primary Gleason grade.

- Nuclear NDRG1 expression was increased in PCa and is positively associated with increasing Gleason grades and clinical stage.

8.3 Strengths and limitations of this study

The work presented in this thesis has a number of strengths. First, the two different cohorts allowed confirmation of the results in samples from two independent patient groups. Second, the sample size of the TMA cohort was large (16 NP and 80 PCa) and comparable to many studies investigating these biomarkers in normal and malignant prostate. For example, key recent studies had a sample size of 103 PCa vs 35 BPH (Ipekci *et al.*, 2015) or 80 PCa patients (Symes *et al.*, 2013) and sometimes better than other studies, such as this study with (10 PCa vs 10 NP) (Zhong *et al.*, 2012). In addition, a second independent antibody and/ or mRNA probe was used to validate the expression patterns of three interesting proteins, Sox7, Sall4 and Zscan4. This gave greater confidence that the staining was accurately reflecting the expression of the target protein.

The study has some limitations that should be considered in future work aimed at extending these findings. A key limitation is that the Bath cohort consists of a small number of samples (34 PCa vs 5 NP) and the TMA cohort lacks clinical data regarding PCa relapse and non-relapse. This means that any conclusions from this work regarding a link to relapse should be treated as preliminary findings. In addition, the TMA used had two cores per sample and a previous comparison between TMAs and whole sections suggested that the optimal number of tissue cores in a TMA which may predict the outcome of the whole tissues of PCa is about 3-4 cores (Rubin *et al.*, 2002). Another limitation is the amount to validation carried out for the antibodies. For three markers a second antibody or RNAscope® were used to validate the staining pattern, but several other methods can be used to validate antibodies (Uhlen *et al.*, 2016). Knockouts are perhaps the most robust method of validation and can help support the use of a particular antibody. This method can not be used in human tissues, but could be carried out in human cell lines that can then be embedded and used for IHC staining. Human knockout cell lines were generated by Horizon Company https://www.horizondiscovery.com/ for the following proteins: Sall4

(HZGHC57167), Zscan4 (HZGHC201516), Sox7 (HZGHC83595), ABCG2 (HZGHC9429) and ALDH1A1 (HZGHC216). This would improve the confidence that the staining patterns shown by IHC accurately reflect the expression of the protein. Knockout cell lines could also be used to validate the RNAscope® staining. Taken together, although there are limitations, this work represents an important step in improving understanding of the molecular basis of PCa formation and progression. Further investigation could investigate the functions of the proteins and establish if any of them could represent a potential diagnostic biomarker for PCa.

8.4 Future work

This section focuses on the four key findings described above and presents a number of suggestions for future work stemming from the investigations conducted here.

In this study, the expression of three potential biomarkers Sox7, ABCG2 and ALDH1A1 were negatively associated with a risk of biochemical relapse in the Bath cohort. However, the sample size in this cohort is small, so it will be very interesting to repeat staining of these proteins with a TMA cohort which has clinical information about relapse such as the one used in this study (Nariculam *et al.*, 2009) which has 82 PCa samples (41 relapsed vs 41 non- relapsed). If the association with relapse was confirmed the functional roles of the three proteins in relapse could then be addressed. It would be very interesting to study the effect of siRNA mediated silencing of these proteins on proliferation and colony formation of PCa cell lines such as PCa C4-2 or LNPCa cell lines. MTS and colony formation assays may be used to detect cell proliferation and colony formation in cultured PCa cell lines, respectively as previously carried out (Liu and Shan, 2016). Additionally, the use of xenograft models, for example using nude mice (Stovall, et al., 2013), would be useful to address the function of these potential biomarkers.

Future research is very important to determine the expression patterns of Sall4 and Sox7, β-catenin and ABCG2 at protein and mRNA levels using IHC and RNAscope® in a large TMA cohort from US Bio max (HproA180PGO5), which consists of 90 PCa and 90 adjacent NP tissues. The current array used in this work only had 16 NP cases, so the new array would allow confirmation of the link between expression of

these proteins and tumour formation. It would be interesting to try and detect soluble Sall4, β-catenin and ABCG2 and NDRG1 levels in serum and/or urine and correlate this result with clinical parameters such as different grade and stage of PCa. This has been carried out for PSA (Kawakami *et al.*, 2004; Lilja *et al.*, 2008; Dunn and Kazer, 2011) and Sox7 (Zhong *et al.*, 2012) in PCa patients as well as for Sall4 in hepatocellular carcinoma (Han *et al.*, 2014) using serum samples. In addition, a recent study on breast cancer has suggested that Sox7 may have a role in regulating Wnt/ β-catenin signalling through its co cooperation with Axin (Liu *et al.*, 2016), so it would be very interesting to investigate an association between Sox7 and Axin in prostate samples from both cohorts, using IHC and RNAscope®.

Previous experiments on NDRG1 in human pancreatic cancer (Kovacevic *et al.*, 2013) suggests it may play a role in regulating the PI3K pathway. For this reason, it would be interesting to find if there is an association between these potential biomarkers and PTEN as a main regulator of the PI3K pathway. This could be carried out using IHC, PCR and/or RNAscope®.

Finally, it will be important to validate the expression patterns of potential biomarkers such as β-catenin, ABCG2, NDRG1, ALDH1A1 and Sox7, that were stained with a single antibody, using either a second independent antibody (IHC) and/ or mRNA probe (RNA scope®).

8.5 Final conclusion

This research suggests that seven of the candidate biomarkers analysed here may play a role in prostate tumour formation, aggressiveness and/or relapse and warrant further investigation to understand their functions and establish if any of them could represent potential diagnostic/prognostic biomarkers for PCa.

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Publications and conference presentations

1. **Dhafer A. Alghezi, Paul Whitley, Mark Beresford, Rebecca Bowen, John Mitchard, Andrew Chalmers. Expression of SALL4 in prostate cancer** [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2018; 2018 Apr 14-18; Chicago, IL. Philadelphia (PA): AACR; Cancer Res 2018; 78 (13 Suppl): Abstract nr 2637. (Abstract published).
2. **Expression of Sall4 in prostate cancer.** American Association of Cancer Research (AACR) annual conference, Chicago, USA (2018). Presented poster.

3. **Prostate cancer stem cell: Potential new biomarkers.** European Cancer Stem Cell Research Symposium - Cancer Stem Cells, Resistance and Tumour Heterogeneity (2nd Research Symposium), Cardiff, UK (2015). Presented poster.
4. **Expression of Sox7 and Zscan4 in prostate cancer.** 12TH CR@B Symposium Event, Bath University, Bath, UK (2015). Presented poster.
5. **Expression of Sall4 and β- catenin in prostate cancer.** Second International Antibody Validation Meeting, University of Bath. Bath city, UK (2016). Presented poster.
6. **Expression of Sall4 and Zscan4 in prostate cancer.** 1st International Cancer Research at Bath (CR@B) conference at Bath University, Bath/ UK (2017). Presented poster.

7. **Decreased Sox7 expression in prostate cancer and negatively associated with increasing Gleason grade and biochemical recurrence.** 60th Annual Meeting of the Italian Cancer Society, Care and Cure of cancer patients: bridging basic research into clinical setting, Milan, Italy, (2018). Presented poster.
8. **Decreased Sox7 expression in prostate cancer and negatively associated with increasing Gleason grade and biochemical recurrence.** 10th Annual Next Generation Sequencing and Clinical Congress, 6th Annual Signal Cell Analysis Congress, 4th Annual Genome Editing Congress and Synthetic Biology Congress Novotel London West, London, UK (2018). Presented poster.